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MODELLING OF LEUKEMIC FUSION GENES IN HUMAN EMBRYONIC STEM CELLS:

INTEGRATED GENOMIC AND BIOLOGICAL STUDY OF THE EFFECTS OF THE FUSION GENE *MLL-AF9*

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CERTIFICA:

Que Don **Jarosław K. Sochacki** ha realizado el presente trabajo: **"Modelling of Leukemic Fusion Genes in Human Embryonic Stem Cells: Integrated Genomic and Biological Study of the Effects of the Fusion Gene *MLL-AF9*"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **grado de Doctor**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo su dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que se conste se extiende el presente certificado,
Madrid, Marzo de 2013

VºBº del Director de la Tesis:

Juan C. Cigudosa

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CERTIFICA:

Que Don **Jarosław K. Sochacki** ha realizado el presente trabajo: **"Modelling of Leukemic Fusion Genes in Human Embryonic Stem Cells: Integrated Genomic and Biological Study of the Effects of the Fusion Gene *MLL-AF9*"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **grado de Doctor**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo su supervisión, autorizando su presentación ante el Tribunal Calificador.

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Eva Mª Richard Rodríguez

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Ever tried, ever failed, no matter,

Try again, fail again, fail better.

Samuel Beckett

Dedicated to my Parents, my Brothers and my whole Family

who did not see me much during the last years.

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"Sólo un exceso es recomendable en el mundo: el exceso de gratitud"

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ABBREVIATIONS

AF4 (MLLT2)	ALL1-Fused gene from chromosome 4 (Mixed Lineage Leukemia Translocated to 2)
***AF9* (*MLL*T3)**	ALL1-Fused gene from chromosome 9 (Mixed Lineage Leukemia Translocated to 3)
AGM	Aorta-Gonad-Mesonephros
AgM	Aggregation Medium
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic leukemia
AT-hook	DNA-binding motif
ATM	Ataxia Telangiectasia Mutated Gene
BCR	Breakpoint Cluster Region
bFGF	Basic Fibroblast Growth Factor
BMP4	Bone Morpho-genetic Protein 4
bp	Base pair
BSA	Bovine Serum Albumine
CBFA1 (RUNX1)	Runt-related transcription factor 1
CBP	CREB-Binding Protein
CDK9	Cyclin-Dependent Kinase 9
cDNA	Complementary DNA
CFC	Colony Forming Cell
CFU	Colony Forming Unit
CIN	Chromosome Instability
CLL	Chronic Lymphoblastic Leukemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukemia
CMML	Chronic myelomonocytic leukemia
CS1, 2	Cleavage Site 1, 2
CYP33	Cyclophilin 33
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMT	DNA methyltransferase
DNA	Deoxyribonucleic Acid
Dot1	DOT1-like histone methyltransferase
EDTA	Ethylenediaminetetraacetic Acid

ENL (MLLT1)	Mixed Lineage Leukemia Translocated to 1
EPO	Erythropoietin
FAB	French-American-British classification of leukemias
FACS	Fluorescence-Activated Cell Sorting
FL	FLT3 Ligand
FLT3	Fms-Like Tyrosine kinase 3
FLT3-ITD	*FLT3*-Internal Tandem Duplication
FLT3-WT	*FLT3*-Wild Type
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
GMP	Granulocyte-Macrophage Progenitor
GSK3	Glycogen Synthase Kinase 3
HBS	HEPES-Buffered Saline
HDAC1, 2	Histone Deacetylase 1, 2
hEB	Human Embryonic Body
hESC	Human Embryonic Stem Cell
HIV-1	Human Immunodeficiency Virus 1
HOX	Homeobox
HPC	Hematopoietic Progenitor Cell
HPC2	Histone Promoter Control protein 2
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem/Progenitor Cell
IL-3	Interleukin 3
IL-6	Interleukin 6
IM	Induction Medium
IVF	In Vitro Fertilization
kDa	kilo Dalton
KO-DMEM	Knock-out DMEM
KO-SR	Knock-out Serum Replacement
LOH	Loss Of Heterozygosity
LSC	Leukemic Stem Cell
LT-HSC	Long-Term HSC
MDS	Myelodysplastic Syndrome
MEIS1	Myeloid Ecotropic Viral Integration Site 1
MLL	Mixed Lineage Leukemia
MLL-PTD	*MLL*-Partial Tandem Duplication
MPP	Multipotent Progenitor Cell
MSC	Mesenchymal Stem Cell
MSC-CM	MSC-Conditioned Medium

MTG	Monothioglycerol
MV 4,11	Human acute monocytic leukemia cell line
NANOG	Homeobox transcription factor
NHEJ	Non-Homologous End Joining
OCT3/4	POU class 5 homeobox gene
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PHD	Plant Homology Domain
PI	Propidium Iodide
PML	Promyelocytic Leukemia
qRT-PCR	Quantitative RT-PCR
RARA	Retinoic Acid Receptor Alpha
REH	Human B cell precursor leukemia cell line
REX1 (Zfp42)	Zinc finger 42 homeobox transcription factor
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PR	Real-time PCR
RTK	Receptor Tyrosine Kinase
SCF	Stem Cell Factor
SEPT	Septin
SET	(Su(var)3-9, Enhancer of zeste, Trithorax)
SIN	Self Inactivating
SNL1, 2	Speckled Nuclear Localization Sites
SOX2	SRY-related homeobox gene
STAT5	Signal Transducer and Activator of Transcription 5
TA	Transcription Activation
tAML	Therapy-related AML
THP1	Human acute monocytic leukemia cell line
TPA	Thrombopoietin
TRA-1-60	Transcription-Associated protein 1-60
TRA-1-81	Transcription-Associated protein 1-81
TRD	Transcription Repression Domain
Trx	Trithorax protein
VEGF	Vascular Endothelial Growth Factor
VSV-G	Vesicular Stomatitis Virus Gene (virus envelope protein)
WHO	World Health Organization

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ABSTRACT

Leukemias harboring the *MLL-AF9* fusion gene are associated with a dismal prognosis and their clinical consequences are well characterized. Concerning the biological processes affected by this fusion gene a little is known and our understanding of its transformation capacities is limited.

Human Embryonic Stem Cells (hESCs) are becoming a powerful tool for modeling human diseases. It is well known, that the process of leukemogenesis manifests as altered cell differentiation. Having this in mind, hematopoiesis-directed differentiation of hESCs is becoming a promising strategy to study the onset of hematopoiesis, especially the emergence of the earliest events leading to the specification of both normal and abnormal hematopoietic tissue (Lensch and Daley 2006).

In our study, we created the first human ESC model for the *MLL-AF9* fusion gene and we then explored the biological and developmental impact of the *MLL-AF9* leukemic fusion gene on the onset of hematopoiesis from hES cells. In addition, we have studied for the first time the effects of the co-expression of the *MLL-AF9* fusion oncogene and a known leukemic secondary hit *FLT3-ITD* on the process of hematopoiesis-directed differentiation of hES cells.

Based on *in vitro* studies, we observed that the expression of the *MLL-AF9* fusion oncogene in hES cells provoked a significant blockage in the process of hematopoietic differentiation of hES cells. Moreover, the *MLL-AF9* fusion gene strongly compromised the clonogenic potential of hematopoietic precursors derived from hESCs. Since the process of *in vitro* hematopoietic differentiation from hESCs closely mirrors early events in embryonic hematopoietic development, the results of our study provide the first indication showing the capacity of the *MLL-AF9* leukemic fusion gene to impair embryonic blood formation when experimentally overexpressed in hESCs. Additionally, we observed that the co-expression of the *MLL-AF9* and the *FLT3-WT/FLT3-ITD* genes in hES cells completely abrogated their hematopoietic potential. These results suggest that the co-expression of the *MLL-AF9* and the *FLT3* genes may exert a synergistic effect on the process of hematopoiesis-directed differentiation of hES cells and lead to the blockage at a very early level of hematopoietic differentiation.

Taken together, our results provide the first indication showing how the leukemic fusion gene *MLL-AF9* when overexpressed in hESCs (separately or together with *FLT3* genes) impairs embryonic blood formation, establishing a potential novel experimental system to further study the developmental impact of the *MLL-AF9* fusion gene.

RESUMEN

Las leucemias caracterizadas por la presencia del gen de fusión *MLL-AF9* están asociadas con un mal pronóstico y sus consecuencias clínicas están bien caracterizadas. Por el contrario, nuestro conocimiento tanto sobre los procesos biológicos afectados por este gen de fusión como sobre sus capacidades de transformación es limitado.

Las células troncales humanas de naturaleza embrionaria (CTE) se están convirtiendo en una herramienta muy poderosa para el modelado de enfermedades humanas. Es bien sabido que el proceso leucémico se manifiesta como alteración de la diferenciación celular. Teniendo esto en mente, la diferenciación hematopoyética de las células troncales humanas se está convirtiendo en una estrategia prometedora para estudiar el inicio de la hematopoyesis, especialmente la aparición de los primeros eventos que conducen a la especificación de tejido hematopoyético normal y maligno (Lensch and Daley 2006). Hemos creado el primer modelo humano de CTE para el gen de fusión *MLL-AF9* y hemos explorado el impacto biológico de la fusión *MLL-AF9* en el proceso de la hematopoyesis a partir de esas células. Además, hemos estudiado por primera vez los efectos de la co-expresión del oncogén de fusión *MLL-AF9* y la mutación ITD del gen *FLT3* en ese mismo proceso de hematopoyesis dirigida. Basándonos en estudios *in vitro*, observamos que la expresión del oncogén de fusión *MLL-AF9* en CTE provoca un bloqueo significativo en el proceso de diferenciación hematopoyética de las células embrionarias. *MLL-AF9* también limita el potencial clonogénico de los precursores hematopoyéticos derivados de las CTE. Dado que el proceso de diferenciación hematopoyética *in vitro* de las CTE refleja estrechamente los estadios tempranos en el desarrollo hematopoyético embrionario, los resultados de nuestro estudio señalan la capacidad de la fusión *MLL-AF9* para impedir la formación de la sangre embrionaria cuando experimentalmente se expresa de forma ectópica en células embrionarias. Además, observamos que la co-expresión de la fusión *MLL-AF9* y los genes *FLT3-WT*/*FLT3-ITD* en las CTE suprimió completamente su potencial hematopoyético, sugiriendo que la co-expresión de la fusión *MLL-AF9* y los genes *FLT3* puede ejercer un efecto sinérgico sobre el proceso de la diferenciación hematopoyética de las CTE y bloquear este proceso en un nivel muy temprano.

En conjunto, nuestros resultados proporcionan la primera indicación sobre cómo el gen de fusión *MLL-AF9* (por separado o junto con *FLT3*) incide negativamente en la formación de sangre embrionaria. Al mismo tiempo, esto establece un novedoso y potente sistema experimental para estudios adicionales de los efectos del desarrollo del gen de fusión *MLL-AF9*.

INTRODUCTION

1. CANCER AS A GENETIC DISEASE

Theodor Boveri is one of the towering figures of twentieth-century genetics, as he was the first to provide a mechanistic basis for the transmission of traits that were proposed by Mendel. In 1914, he suggested that tumors might arise as a consequence of abnormal segregation of chromosomes to daughter cells (Aamot, Micci et al. 2005). The development of cytogenetic techniques was crucial in developing our understanding of the chromosome aberrations that were visualized by Boveri under the microscope. Many concepts that are now commonly accepted were foreshadowed by Boveri, including cell-cycle checkpoints, oncogenes and tumor-suppressor genes, tumor predisposition, and relationship between genetic instability and cancer. It is a sobering thought that the experimental proof of many of these predictions became the cornerstone of cancer research over the past 95 years. (Figure 1-1).

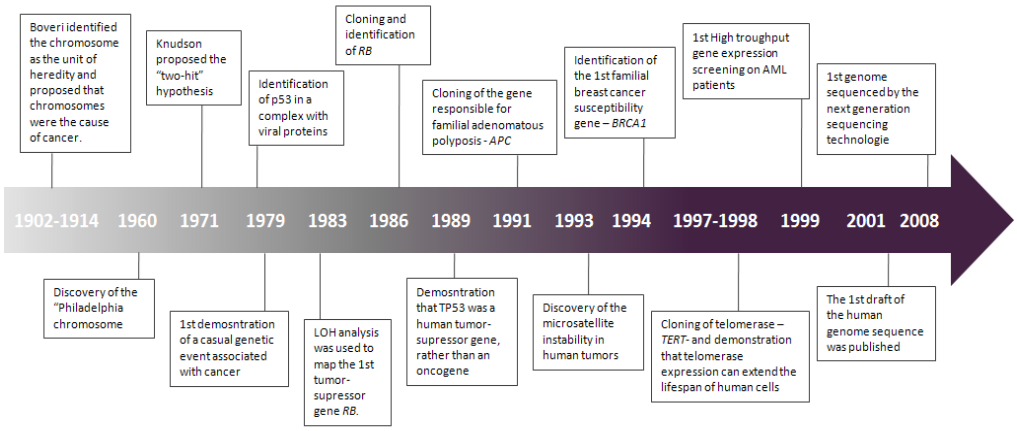

Figure 1-1 | Time course of cancer research (Modified from Balmain 2001).

The medical definition of cancer corresponds to a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood).

In essence, cancer is the most common human genetic disease, involving dynamic changes in the genome (Andersen, Gruszka-Westwood et al. 2005). Tumorigenesis, the process that conducts the transition from a normal human cell into highly malignant derivates, is a multi-step process driven by the acquisition of mutations (any change in the sequence of the genome) over the lifetime that miss the cell's maintenance machinery (Annunziata, Davis et al. 2007). Unlike other types of diseases wherein mutations in one single gene cause disease, cancer cannot be caused by a single gene defect (Andersen, Gruszka-Westwood et al. 2005).

Mutations can generally occur in three different types of genes: oncogenes, tumor-suppressor genes and stability genes (Andersen, Gruszka-Westwood et al. 2005). **Oncogenes** are mutated forms of genes (proto-oncogenes) that cause normal cells to grow out of control, by exhibiting permanently activation when it is not supposed to be, and become cancer cells. Proto-oncogenes are the genes that normally control how often a cell divides and the degree to which it differentiates. The activation of these genes can be due to activating point mutations (*BRAF*), chromosomal translocations (*BCR-ABL*) or gene amplifications (*HER2-NEU*) (Andersen, Gruszka-Westwood et al. 2005). **Tumor-suppressor genes** (TSGs) are normal genes that slow down cell division, repair DNA damage, and regulate the apoptosis process. Inactivation of a tumor-suppressor gene arises by means of mutations that result in a truncated protein, deletions, and insertions of various sizes or from epigenetic silencing. **Stability genes or caretakers** genes, compared to the latest ones, act differently preventing cancer. This class of genes is responsible for the maintenance of DNA content and integrity in the cell. This action is conducted by repairing subtle mistakes occurring during normal DNA replication event or by controlling processes like mitotic recombination and chromosomal segregation (Andersen, Gruszka-Westwood et al. 2005). Inactivation of these genes leads to increase mutation rate in other genes. The complete inactivation of both alleles is needed to result in a physiologic effect for both stability and tumor-suppressor genes.

1.1. TYPES OF GENETIC ABERRATIONS IN CANCER

Attending to the final effect on the gene involved in a genetic aberration, there are two dominating types of genetic pathways that have been identified as cancer initiating factors: the inactivation of genes by any mean (deletion, mutation, epigenetic or miRNA mechanisms) and the activation or deregulation of genes as a result of point mutation, amplification or balanced cytogenetic abnormalities. The accumulation of mutations is widely accepted as the driving force that leads to cancer. But the mechanisms through which these mutations are generated are still under debate (Annunziata, Davis et al. 2007). The types of genetic alterations can be divided into four categories. First, subtle **sequence changes**- these changes involve base substitutions, deletions or insertions of a few nucleotides that cannot be detected through cytogenetic analysis. Second, **balanced chromosome translocations**- alterations caused by rearrangement of parts between nonhomologous chromosomes. This alteration can give rise to a fusion gene, with tumorigenic properties, produced by the junction of two otherwise separated genes. Third, **unbalanced chromosomal**

rearrangements - refers to any change where the affected genome shows a modified number of copies of genetic material (gains or losses). When unbalanced aberrations are the result of structural rearrangements, we are referring to them as gene amplifications (any duplication of a DNA region that contains a gene), amplicons (which contain 0.5-10 mega bases of DNA, and are different from the duplications of much larger chromosome regions that result from aneuploidy and translocations) or homozygous deletions. When unbalanced aberrations are a result of whole chromosome number alterations we are referring to them as aneuploidy. This state is induced by a increased rate of losses or gains of whole chromosomes or large parts of chromosomes during cell division. The consequence is an imbalance in the chromosome number (aneuploidy) and an enhanced rate of loss of heterozygosity (LOH) (Avet-Loiseau, Vigier et al. 1997). The elevated rate of LOH is an important property since it accelerates the inactivation of TSGs (Aviel-Ronen, Coe et al. 2008). The alteration in chromosome number is commonly referred as chromosomal instability (CIN). A major question of cancer genetics is whether the CIN extent, or any genetic instability, is an early event and consequently a driving force for tumor progression or if it is simply an unspecific epiphenomenon that occurs spontaneously. Nevertheless, there are strong evidences, like the presence of aneuploidy in pre-malignant stages, the high frequency rate of CIN in cancer cell lines and the presence of mutations in genes that control chromosome segregation during mitosis, that strongly suggest the important role of aneuploidy in carcinogenic process (Bailly, Bosselut et al. 1994). And finally, **epigenetics changes-** whether genomic instability is due to a genetic change in tumor cells or epigenetics is still up in the air, although all the evidences point out that genetic instability is heritable at the cellular leverl and that such instability is associated with a predisposition to cancer (Balmain 2001). An “explosion” of data indicates that epigenetic status is important, especially those resulting in the silence of key regulator genes. Single mutations are generally not sufficient to cause cancer, but they produce changes that may predispose cells to malignant growth. Additional mutations in other genes, caused by damage from the environment, predispose normal cells to malignant transformation.

Thus, cancer is a multi-step process involving the interaction between genetics and epigenetics in all stages. The accurate identification of the alterations that lead to cancer contributes enormously to the early detection, diagnosis, prognosis and treatment of the disease.

1.2. TUMOURS WITH BALANCED CHROMOSOMAL TRANSLOCATIONS

Early observations that specific recurring chromosome aberrations, such as translocations, were often associated with a particular type of leukemia, lymphoma or sarcoma led researchers to believe that chromosome rearrangements might be involved in cellular transformation (Bandres, Malumbres et al. 2005). Chromosome rearrangements account for a big part of the initiating events that, resulting in gene deregulation, are among the major contributors to the oncogenesis. Molecular characterization of these chromosomal abnormalities has helped to identify a high number of fusion genes involved in cancer. At present, 358 fusion genes involving 337 different genes have been identified in hematological and solid tumors (Baud and Karin 2009). Leukemias and lymphomas are particularly associated with recurrent balanced rearrangements (Table 1-1). Almost every subtype is associated with a particular translocation with remarkable specificity with clinical and biological features (Bentz, Werner et al. 1996; Bandres, Malumbres et al. 2005).

Table 1-1 | Structure and function of some representative genes rearranged in balanced chromosomal aberrations in acute myeloid and lymphoid leukemia (Modified from Pedersen-Bjergaard and Rowley 1994).

Genes	Chromosome band	Structural motifs	Function
MLL (HRX or ALL1)	11q23	AT hook, zinc finger, trithorax homology	DNA binding, gene regulation
AF4 or MLLT2	4q21	Nuclear localization signal	Unknown
AF9 or MLLT3	9p22	Nuclear signalizaton signal	Unknown
ENL or MLLT1	19p13	Nucelar signalization signal	Unknown
AML or CBFA1	21q22	runt homology	DNA binding, gene regulation
EAP	3q26	-	Ribosomal protein
ETO, CDR or MTG8	8q22	Zinc finger	DNA binding, gene regulation
PML	15q22	Zinc finger	DNA binding, gene regulation
RARA	17q12	Zinc finger	DNA binding, gene regulation
MYH11	16p13	α-Helix coiled coil	-
CBFB or PEBP2β	16q22	-	Transcriptional modulator
DEC	6p23	-	Unknown
CAN	9q34	Helix-hoop-helix	DNA binding, gene regulation

1.3. HUMAN LEUKEMIAS: GENERAL CONCEPTS AND CLASSIFICATION

Leukemia (from the Ancient Greek λευκός *leukos* "white", and αἷμα *haima* "blood") is a type of cancer of the blood or bone marrow characterized, among other features by an abnormal increase of immature white blood cells called "blasts". Leukemia is a very broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases affecting the blood, bone marrow, and lymphoid system, which are all known as hematological neoplasms.

Clinically and pathologically, leukemia is subdivided into a variety of large groups. The first clinical division is between its *acute* and *chronic* forms (Table 1-2).

Table 1-2│ Four major types of leukemia.

Cell type	Acute	Chronic
Lymphocytic (lymphoblastic) leukemia	Acute lymphoblastic leukemia (ALL)	Chronic lymphocytic leukemia (CLL)
Myelogenous (myeloid) leukemia	Acute myeloblastic leukemia (AML)	Chronic myeloblastic leukemia (CML)

The most common acute leukemia, affecting adults is acute myeloid leukemia (AML). It is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. Although AML is a relatively rare disease, accounting for approximately 1.2% of cancer deaths in the United States (Jemal, Thomas et al. 2002) its incidence is expected to increase as the population ages.

The symptoms of AML are caused by replacement of normal bone marrow with leukemic cells, which causes a drop in red blood cells, platelets, and normal white blood cells. These symptoms include fatigue, shortness of breath, easy bruising and bleeding, and increased risk of infection. Several risk factors and chromosomal abnormalities have been identified, but the specific cause is not clear. As an acute leukemia, AML progresses rapidly and is typically fatal within weeks or months if left untreated.

AML has several subtypes; treatment and prognosis varies among subtypes. Five-year survival varies from 15–70%, and relapse rate varies from 33–78%, depending on subtype. AML is treated initially with chemotherapy aimed at inducing a remission; patients may go on to receive additional chemotherapy or a hematopoietic stem cell transplant. Recent research into the genetics of AML has resulted in the availability of tests that can predict which drug or drugs may work best for a particular patient, as well as how long that patient is likely to survive.

Two systems have been used to classify AML into subtypes: the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification.

In the 1970s, a group of French, American, and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7 (Table 1-3) (wwww.cancer.org), based on the type of cell from which the leukemia developed and how mature the cells are. This was based largely on how the leukemia cells looked under the microscope after routine staining.

Table 1-3 | FAB classification of AML (Modified from www.cancer.org).

FAB subtype	Name	Cytogenetics	% of adult AML patients	Prognosis compared to average for AML
M0	Undifferenitiated acute myeloblastic leukemia		5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation		15%	Average
M2	Acute myeloblastic leukemia with maturation	t(8;21)(q22;q22), t(6;9)	25%	Better
M3	Acute promyelocytic leukemia (APL)	t(15;17)	10%	Best
M4	Acute myelomonocytic leukemia	inv(16)(p13q22), del(16q)	20%	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	inv(16), t(16;16)	5%	Better
M5	Acute monocytic leukemia	del (11q), t(9;11), t(11;19)	10%	Average
M6	Acute erythroid leukemia		5%	Worse
M7	Acute megakaryoblastic leukemia	t(1;22)	5%	Worse

Subtypes M0 through M5 all start in precursors of white blood cells. M6 AML starts in very early forms of red blood cells, while M7 AML starts in early forms of cells that make platelets. Some subtypes of AML defined in the FAB system are linked with certain symptoms. For example, bleeding or blood clotting problems are often a problem for patients with the M3 subtype of AML, also known as acute promyelocytic leukemia (APL). Identifying APL is very important for 2 reasons. First, certain complications of APL can often be prevented by appropriate treatment. Second, APL is treated differently from most other forms of AML: it usually responds to retinoids (drugs related to vitamin A).

The FAB classification system is useful and is still commonly used to group AML into subtypes. But it does not take into account many of the factors that are known to impact prognosis (outlook). The World Health Organization (WHO) has proposed a newer system that includes some of these factors to try to help better classify cases of AML based on a patient's outlook. The WHO classification system divides AML into several broad groups (Table 1-4) (www.cancer.org).

Table 1-4 | WHO classification of AML (Modified from www.cancer.org).

Name	Description
Acute myeloid leukemia with recurrent genetic abnormalities	- AML with a translocation between chromosomes 8 and 21 - AML with a translocation or inversion in chromosome 16 - AML with translocations in rearrangements involving the *MLL* gene - APL (M3), which usually has translocation between chromosomes 15 and 17
Acute myeloid leukemia with multilineage dysplasia	This category includes patients who have had a prior myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD) that transforms into AML. This category of AML occurs most often in elderly patients and often has a worse prognosis.
Acute myeloid leukemia related to previous chemotherapy or radiation	This category includes patients who have had prior chemotherapy and/or radiation and subsequently develop AML or MDS. These leukemias may be characterized by specific chromosomal abnormalities, and often carry a worse prognosis.
Acute myeloid leukemia not otherwise specified (includes cases that do not fall into one of the above groups, similar to the FAB classification)	- Undifferentiated AML (M0) - AML with minimal maturation (M1) - AML with maturation (M2) - Acute myelomonocytic leukemia (M4) - Acute monocytic leukemia (M5) - Acute erythroid leukemia (M6) - Acute megakaryoblastic leukemia (M7) - Acute basophilic leukemia - Acute panmyelosis with fibrosis - Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)
Undifferentiated or biphetotypic leukemias	Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukemias

1.3.1. *MLL* LEUKEMIAS

Leukemias that bear translocations involving the *MLL* gene on chromosome 11q23 possess unique clinical and biological characteristics and, as such, have been categorized as a unique entity in the WHO classification. *MLL* rearrangements are found in >70% of infant leukemias, whether the immunophenotype is more consistent with ALL or AML (Biondi, Cimino et al. 2000), but are less frequent in leukemias from older children. *MLL* translocations are also found in approximately 10% of AMLs in adults, and in therapy-related leukemias (t-leukemias) that develop in patients previously treated with topoisomerase II inhibitors for other malignancies. The t-leukemias are most often characterized as AML (tAML), but can be acute lymphoblastic leukemias (tALL), myelodysplastic syndrome (tMDS) or chronic myelomonocytic leukemias (tCMML). Overall, leukemias that bear *MLL* rearrangements are found in approximately 10% of human leukemias (Huret, Dessen et al. 2001). More than 100 different translocation fusion partners have been identified; however, a subset account for most cases. The five most frequent *MLL* rearrangements, accounting for approximately 80% of all *MLL*-translocation-bearing leukemias, are: t(4;11)(q21;q23) or *MLL–AF4*; t(9;11)(p22;q23) or *MLL–AF9*; t(11;19)(q23;p13.3) or *MLL–ENL*; t(10;11)(p12;q23) or *MLL–AF10*; and t(6;11)(q27;q23) or *MLL–AF6* (Huret, Dessen et al. 2001; Meyer, Schneider et al. 2006) (Figure 1-2).

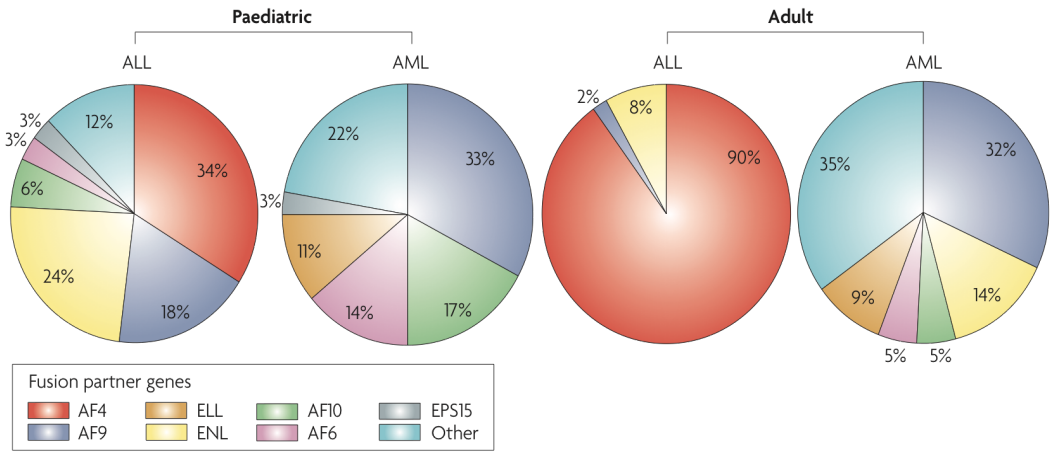

Figure 1-2 | Distribution of major *MLL* fusion partner genes in de novo childhood and adult leukemias (Modified from Krivtsov and Armstrong 2007).

Patients with *MLL*-rearranged ALL have a particularly poor outcome compared with children with other forms of ALL (Chen, Sorensen et al. 1993) and *MLL*-rearranged leukemias that occur after treatment with topoisomerase II inhibitors have also a similarly poor prognosis. *MLL*-rearranged AMLs possess a similar prognosis as other AMLs (Felix, Hosler et al. 1995; Mrozek, Heinonen et al. 1997; Rubnitz, Raimondi et al. 2002). The association of *MLL* translocations with a young age at diagnosis, the presence of *MLL* translocations in both ALL and AML, and the poor clinical outcome of patients with *MLL* fusions have generated much interest in the biology of *MLL*-translocation-associated leukemias.

1.3.1.1. *MLL* GENE STRUCTURE

The Mixed Lineage Leukemia (*MLL*) gene, located on chromosome 11 (11q23) (Figure 1-3) is approximately 89 kb long, consists of 37 exons (Tkachuk, Kohler et al. 1992), and encodes a 3,969 amino acid nuclear protein with a complex domain structure.

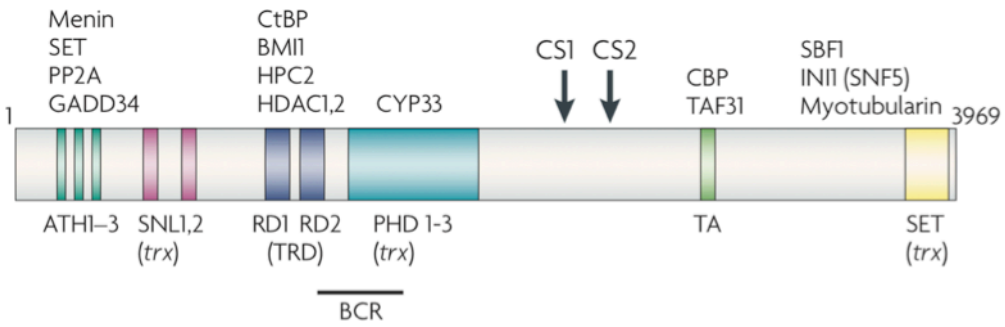

Figure 1-3 | Structure of the *MLL* gene (Modified from Krivtsov and Armstrong 2007).

The mature MLL protein consists of two non-covalently associated subunits (MLL^N (300 kDa) and MLL^C (180 kDa)) produced by cleavage of nascent MLL by taspase 1 after amino acid residues 2,666 (cleavage site 1 (CS1)) and 2,718 (CS2) (Daser and Rabbitts 2004). Proteins that bind to specific domains are noted above each domain. The N terminus contains three short AT-hook motifs (ATH 1–3), which are thought to mediate binding to the minor groove of AT-rich genomic DNA sequences (Zeleznik-Le, Harden et al. 1994). There are two speckled nuclear localization sites (SNL1 and SNL2) immediately C-terminal to the AT-hooks that are followed by a transcriptional repression domain (TRD) consisting of two functional subunits, RD1 and RD2. RD1 contains a DNA methyltransferase (DMT) homology domain that includes a CxxC zinc-finger motif that may recruit proteins such as HPC2 and the transcriptional

co-repressor CtBP (Xia, Anderson et al. 2003). RD2 recruits histone deacetylases HDAC1 and HDAC2 (Xia, Anderson et al. 2003). The plant homology domain (PHD) zinc-finger motifs may mediate binding of the cyclophilin, CYP33, and potentially other proteins (Fair, Anderson et al. 2001). The transcriptional activation (TA) domain recruits the transcriptional co-activator CBP (CREB-binding protein) and precedes a C-terminal SET (Su(var)3-9, enchancer-of-zeste, trithorax) domain that possesses histone H3 lysine 4 (H3K4) methyltransferase activity (Milne, Briggs et al. 2002; Nakamura, Mori et al. 2002) and is structurally homologous to Drosophila melanogaster trithorax. The breakpoint cluster region (BCR) spans exons 8–13.

1.3.1.2. *MLL* GENE FUNCTION

The mixed lineage leukemia protein MLL, the human homolog of the Drosophila trithorax protein (Trx), was originally identified through its association with acute lymphoid and myeloid leukemias (Xia, Popovic et al. 2005; Terranova, Agherbi et al. 2006). *MLL* is essential for both embryonic development and normal hematopoiesis through its regulation of the clustered homeobox (*HOX*) genes and other genes important for developmental regulation (Figure 1-4) (Dou and Hess 2008).

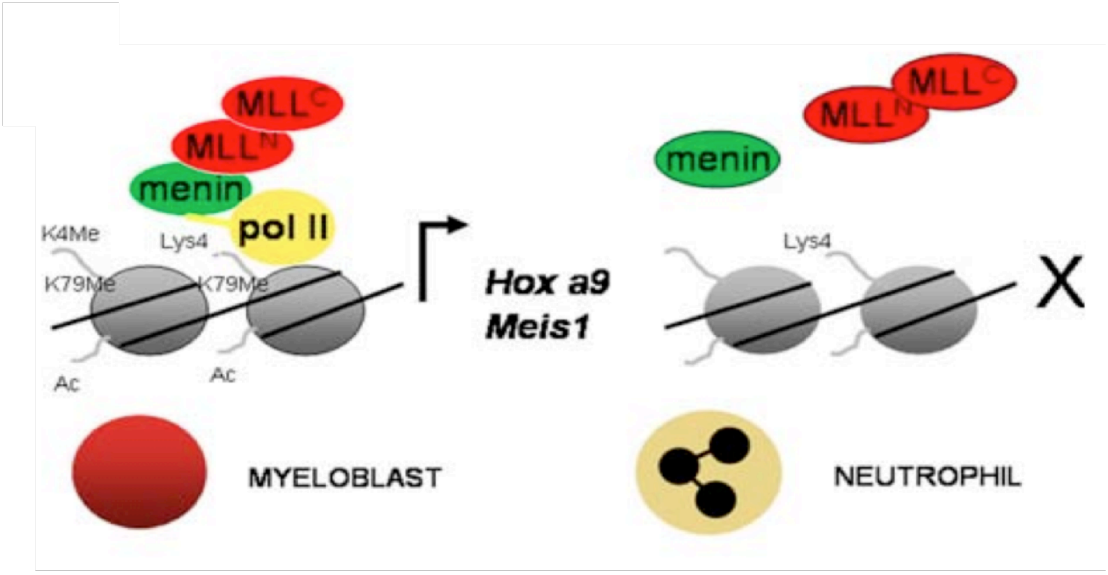

Figure 1-4 | Schematic regulation of transcription by *MLL* methyltransferase complexes in hematopoietic cells (Modified from Dou and Hess 2008).

The MLL methyltransferase complex is likely to be recruited to RNA polymerase II at target genes through interactions with menin (other MLL interacting proteins are not shown in the figure). MLL has intrinsic lysine 4-methyltransferase activity and, in addition, MLL recruits histone acetyltransferases to its target loci. Upon differentiation

of the myeloblasts into neutrophils (right panel), both menin and MLL localization appears to be lost. This is accompanied by a marked downregulation in histone marks on the histone tails.

Knockout mouse models provide clear evidence that *MLL* plays a major role in regulation of hematopoiesis. *MLL* null mouse embryos show defects in both fetal liver and yolk sac hematopoiesis (Hess, Yu et al. 1997; Yagi, Deguchi et al. 1998). These defects are due to reduced number of hematopoietic progenitor cells and the delayed onset of differentiation instead of blocking specific lineages. Conventional *MLL* knockout mice show pleiotropic effects and are early embryonic lethal (Yu, Hess et al. 1995). Studies performed using chimeric fetal and adult animals reconstituted with *MLL* deficient ES cells showed multi-lineage blocks in lymphopoiesis including the complete loss of B, T and NK cells (Ernst, Fisher et al. 2004). *MLL* also appears to be involved in immune regulation as specific loss of memory T helper type2 cells (Th2) is seen in adult *MLL* haploinsufficient (*MLL*+/-) mice (Yamashita, Hirahara et al. 2006).

1.3.1.3. *MLL* FUSION PROTEINS

Much has been speculated about the origins of the chromosomal aberrations that convert an innocuous chromatin modifier into a pernicious oncogene. Several lines of evidence point to a mishap in non-homologous end joining of double strand breaks as the most likely reason for 11q23 translocations (Dou and Hess 2008).

For one, the characteristic peak of mixed lineage leukemia in patients treated with etoposide is highly suggestive for an involvement of DNA double strand lesions in the etiology of *MLL* fusions. Etoposide inhibits topoisomerase II and therefore causes breaks in both DNA strands. Indeed, it could be shown that the locus 11q23 is particularly susceptible to this kind of assault in cells treated with topo II inhibitors (Libura, Slater et al. 2005; Hars, Lyu et al. 2006). Alternatively, a break might be introduced at early stages of apoptotic DNA fragmentation that was later aborted and repaired. Published data also provide some support for this scenario, as breaks preferentially occur at 11q23 in early apoptotic cells (Betti, Villalobos et al. 2001).

Whatever the reason for the initiating event, an aberrant non-homologous end joining (NHEJ) process most likely causes the cross-wise sealing of the DNA ends. A close examination of the breakpoint junctions revealed that they frequently code for non-templated nucleotides (Gillert, Leis et al. 1999), a hallmark of NHEJ repair as known from generation of antibody and T-cell receptor diversity. Despite the attractions of this hypothesis as an explanation for the origin of 11q23 translocations, it does not take into consideration that many double strand breaks induced by background

radiation are continuously repaired in each cell without dire consequences. In this respect, a publication might be important showing that double strand breaks lead to chromosomal aberrations only in cells with impaired ATM-dependent DNA-damage signaling, whereas normal cells are able to join free ends correctly (Nakada, Katsuki et al. 2006).

The 8.3 kb breakpoint cluster region between exons 8 and 13 (Figure 1-5) is the target for most *MLL* rearrangements (Ayton and Cleary 2001) (Figure 1-3), and contains topoisomerase II cleavage sites along with nuclear matrix attachment regions that are likely to contribute to the mechanism by which translocations occur. Deregulation of these processes may lead to interchromosomal translocations found in leukemias (Strissel, Strick et al. 1998). The rearrangements always occur such that an in- frame chimeric protein is produced. All identified *MLL* fusions contain the first 8–13 exons of *MLL* and a variable number of exons from a fusion partner gene (FPG) (Figure 1-5) (Dou and Hess 2008).

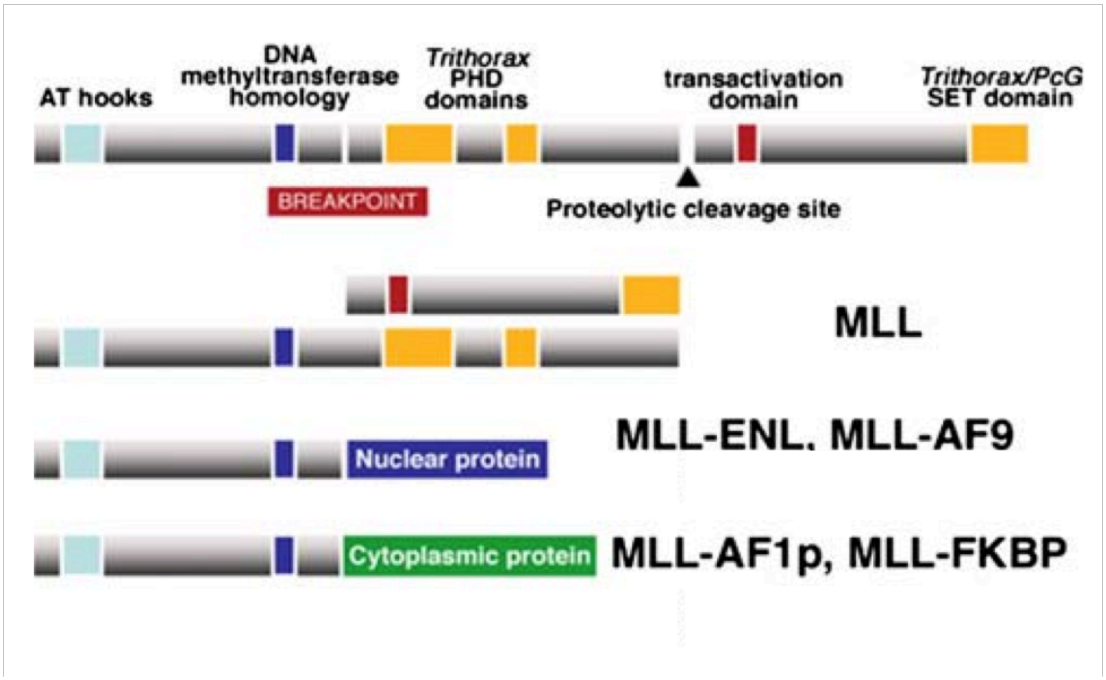

Figure 1-5 | Schematic representation of the *MLL* gene and the two general types of MLL fusion proteins (Modified from Dou and Hess 2008).

The first and most striking property of MLL fusion proteins is their incredible diversity. *MLL* has been found in 100 different translocations and, at least, 54 partner genes have been cloned (http://atlasgeneticsoncology.org/Genes/*MLL*.html). Despite this variety, most cases of mixed lineage leukemia present as a clinical entity and gene expression signatures in leukemic blasts do not separate *MLL* fusions according to the

fusion partner (Armstrong, Staunton et al. 2002; Yeoh, Ross et al. 2002; Ferrando, Armstrong et al. 2003; Ross, Zhou et al. 2003). Therefore, it was a long standing question how a multitude of different proteins could cause the same disease. Two facts gave early clues this problem. Firstly, all *MLL* fusion proteins share a common structure with the respective partners invariably fused in frame to MLL^N right after the CxxC domain but excluding the PHD fingers. Secondly, proteins joined to *MLL* clearly fall into two classes. Only 6 frequent partner proteins (AF4, *AF9*, ENL, AF10, ELL, AF6) constitute the bulk (> 85%) of all clinical cases of mixed lineage leukemia (Burmeister, Meyer et al. 2009; Meyer, Kowarz et al. 2009) (Table 1-5) whereas the remaining fusions were cloned each from a few isolated, mostly adult patients.

Table 1-5 | Classification of *MLL* fusion partners (Modified from Krivtsov and Armstrong 2007).

Putative function		Chromosome	Fusion partner	Frequency in leukemia
Group 1	Nuclear, putative DNA-binding proteins	4q21 9p23 19p13.3 10p12 19p13.1	*AF4* *AF9* *ENL* *AF10* *ELL*	>80%of *MLL*-rearranged leukemias
Group 2	Cytoplasm, presence of coiled-coil oligomerization domain	1q32 17p13 19p13 6q27 Xq13	*EPS15* *GAS7* *EEN* *AF6* *AFX*	>10%
Group 3	Cytoplasm, septin family, interact with cytoskeletal filaments, have a role in mitosis	Xq22 22q11 Xq24 17q25 4q21	*SEPT2* *SEPT5* *SEPT6* *SEPT9* *SEPT11*	>1%
Group 4	Nuclear, histone acetyltransferases	16q13 22q13	*CBP* *P300*	>1%
Group 5	*MLL* parial tandem duplication of exons 5-11 (*MLL-PTD*)	11q23	N/A	4-7% of all AML with normal karyotype

AML, acute myeloid leukemia; CBP, CREB binding protein; *MLL*, mixed lineage leukemia,; N/A, not applicable; PTD, partial tandem duplication

This distinction is mirrored by the biology of the respective proteins. With the exception of AF6, all frequent *MLL* partners are nuclear while cytoplasmatic localization predominates amongst the rarely occurring *MLL* fusions. Therefore it was expected that at least two different mechanisms should be responsible for *MLL* fusion function. Further clarification of these pathways was promoted by the development of an in vitro

assay that was able to measure the biological readout of *MLL* fusion activity (Lavau, Szilvassy et al. 1997). This serial replating assay records an inhibition of hematopoietic differentiation as surrogate parameter for transformation activity. A block in differentiation can be visualized as enhanced clonogenic capacity of hematopoietic precursor cells after repeated replating in semisolid medium. With respect to the *MLL* portion included in the fusions, deletion studies demonstrated that the LEDGF-menin binding motif and the CxxC domain were absolutely necessary for the overall function of *MLL* fusions (Slany, Lavau et al. 1998; Ayton, Chen et al. 2004). In addition, it was mandatory that the breakpoint in *MLL* was upstream of the PHD fingers because artificial *MLL* fusions including this domain lost their transforming capacity (Chen, Santillan et al. 2008; Muntean, Giannola et al. 2008). This explains the strict conservation of the fusion breakpoints found in leukemic blasts.

1.3.1.4. MECHANISMS OF *MLL* INDUCED TRANSFORMATION

Mixed lineage leukemia translocation partners are very diverse, however, all the *MLL* fusion proteins examined to date upregulate expression of *HOXA9* and *MEIS1*, and this appears to be pivotal for leukemogenesis (Dou and Hess 2008). *HOX* genes including *HOXA9* and *A9* and the *HOX* cofactor *MEIS1* are normally only expressed in early Sca1+ Lin- hematopoietic stem cells and then their expression is rapidly downregulated (Lawrence, Sauvageau et al. 1996; Magli, Largman et al. 1997; Pineault, Helgason et al. 2002; Zeisig, Milne et al. 2004). Although *MLL* is expressed throughout hematopoietic differentiation, normally *HOX* gene and *MEIS1* expression is physiologically downregulated. In the presence of *MLL* fusion proteins, this mechanism is perturbed (Figure1-6).

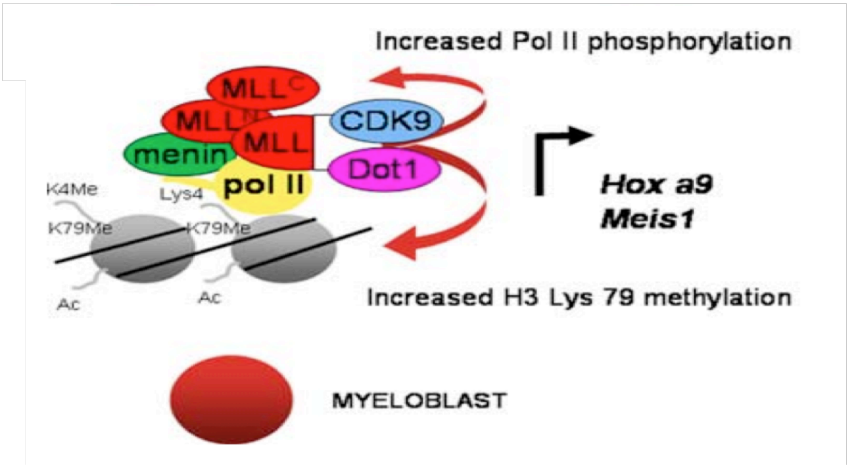

Figure 1-6 | Schematic representation of transcriptional deregulation by MLL fusion proteins involving nuclear translocation partners (Modified from Dou and Hess 2008).

Increasing evidence suggests that the most common *MLL* translocation such as *AF4*, *ENL*, and *AF9* interact with the complex, which includes CDK9. In addition, abundant experimental evidence supports a role for Dot1 in *MLL* mediated transcriptional deregulation. The CDK9 results in increase RNA polymerase II phosphorylation, while the Dot1 increases histone H3 lysine 79 methylation at target promoters. The ultimate result is an overexpression of *HOXA9* and *MEIS1*, which is normally downregulated during hematopoietic differentiation, resulting in immortalization and transformation.

In keeping with this, human leukemias with *MLL* rearrangements, either lymphoid or myeloid, consistently express *HOXA9*, *HOXA9* and *MEIS1* (Rozovskaia, Feinstein et al. 2001; Armstrong, Staunton et al. 2002; Yeoh, Ross et al. 2002). Experimental models provide strong evidence that upregulation of *HOX* genes, particularly *HOXA9* and *MEIS1*, accounts for *MLL* fusion protein leukemogenicity. *HOXA9* and *HOXA9* are consistently expressed in leukemias arising in BXH2 as a result of retroviral integration (Moskow, Bullrich et al. 1995; Nakamura, Largaespada et al. 1996). Notably, more than 95% of leukemias with *HOXA9* and *A9* over expression show a second integration resulting in over expression of *MEIS1*. Co-transduction of *HOXA9* and *MEIS1* immortalizes hematopoietic progenitors in vitro and rapidly accelerates leukemia development in transplanted mice (Kroon, Krosl et al. 1998; Zeisig, Milne et al. 2004). These results are further supported by the inability of *MLL* fusion proteins to transform *HOXA9* knockout bone marrow (Ayton and Cleary 2003). In addition, overexpression of *HOXA9* and *MEIS1* has been implicated in other hematologic malignancies including AML without *MLL* rearrangements (Golub, Slonim et al. 1999; Quentmeier, Dirks et al. 2004). AML and ALL with the *AF10- CALM* translocation (Dik, Brahim et al. 2005), AML with *HOX-NUP* (Dorsam, Ferrell et al. 2004) gene fusions (Calvo, Sykes et al. 2002) and progression of CML to blast crisis (Tedeschi and Zalazar 2006).

Undoubtedly, *HOX* deregulation is the most important factor for *MLL* fusion induced leukemogenesis (Ernst, Mabon et al. 2004; Zeisig, Milne et al. 2004; Guenther, Jenner et al. 2005; Horton, Grier et al. 2005; Milne, Dou et al. 2005; Milne, Martin et al. 2005; Wong, Iwasaki et al. 2007; Erfurth, Popovic et al. 2008; Li, Luo et al. 2009; Somervaille, Matheny et al. 2009). HOX proteins, especially HOXA9, and its dimerization partner MEIS1, are major hematopoietic oncoproteins that are overexpressed in a wide variety of different leukemias and that act, at least partially, through activation of the protooncogene c-Myb (Hess, Bittner et al. 2006). In general, *HOX* transcription factors are not only master controls of embryonic development but they also direct normal hematopoietic differentiation. *HOX* expression is high in stem cells and early precursors and needs to be downregulated for maturation. Therefore, a

continuous ectopic *HOX* expression will block differentiation and create a rapidly proliferating pre-leukemic precursor pool (Figure 1-7). Secondary mutations will have to occur to convert this smoldering state into an acute leukemia. Such mutations have been found in murine experimental models (Horton, Walf-Vorderwulbecke et al. 2009) and also in patient cells that frequently carry an activating mutation in the receptor tyrosine kinase Flt-3 (Brown, Levis et al. 2005; Stam, Schneider et al. 2007). In a very surprising development, it has also recently been suggested that increased glycogen-synthase-kinase 3 activity is involved in the etiology of mixed lineage leukemia, an unexpected finding because GSK3 normally is a tumor suppressor gene (Wang, Smith et al. 2008).

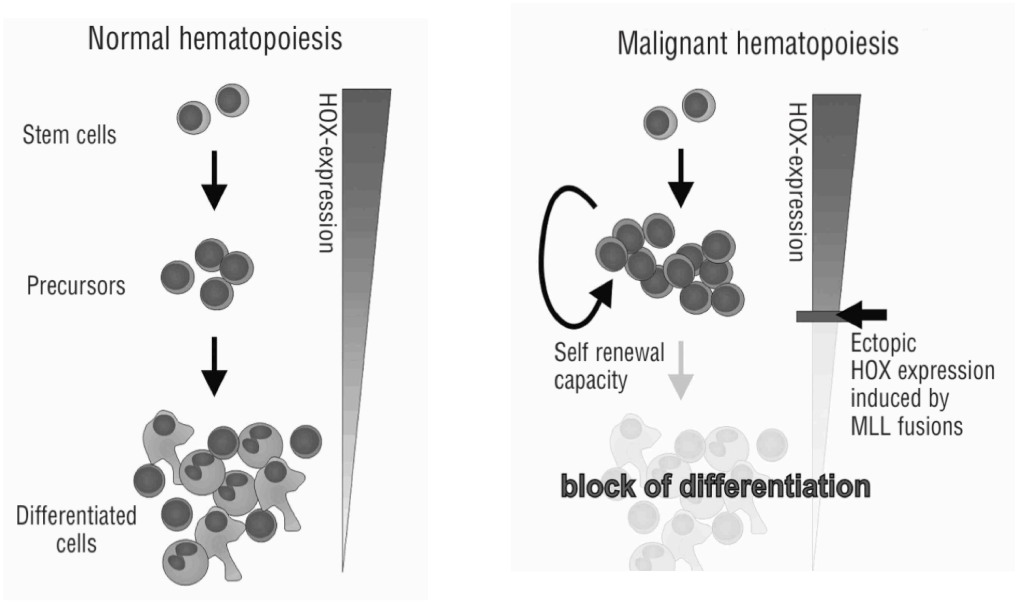

Figure 1-7 | The role of HOX proteins in the control of hematopoiesis (Modified from Slany 2009).

It is very interesting that mixed lineage leukemia tends to be a pediatric disease in contrast to many other tumors that arise later in life, because several years are needed to accrue the mutations necessary to convert a normal cell into a cancerous state. An attractive hypothesis to answer this question has been brought forward by Greaves and colleagues (Eguchi, Eguchi-Ishimae et al. 2006). They speculated that a persistent genetic assault during gestation first produces *MLL* translocations. Once these are transcribed, the presence of the fusion proteins might sensitize cells to further mutations induced by the same mutagens that created the fusion before. In this way, secondary events would accumulate very rapidly and congenital leukemia would ensue.

1.3.2. *MLL-AF9* LEUKEMIAS

The translocation t(9;11)(p22;q23) (Figure 1-8) (www.atlasgeneticsoncology.org) is the most common of the 11q23 alterations in pediatric de novo acute myeloid leukemia (AML) (Creutzig, Zimmermann et al. 2005; Ravindranath, Chang et al. 2005; Smith, Alonzo et al. 2005) and in therapy-related AML following treatment with topoisomerase II inhibitor drugs (Langer, Metzler et al. 2003). This translocation is not usually detected in pediatric acute lymphoblastic leukemia (ALL) but it is the third more frequent abnormality reported in infant-ALL (Pieters, Schrappe et al. 2007). In addition, most infant acute leukemias show *MLL* rearrangements, including t(9;11). In AML, it is frequently associated with FAB subtypes with monocytic features, M4 or M5. Several authors have reported that childhood AML with *MLL-AF9* may have a better outcome (Rubnitz, Raimondi et al. 2002; Smith, Alonzo et al. 2005), although this is not supported by other reports; in pediatric ALL it is associated with poor prognosis (Pui, Gaynon et al. 2002).

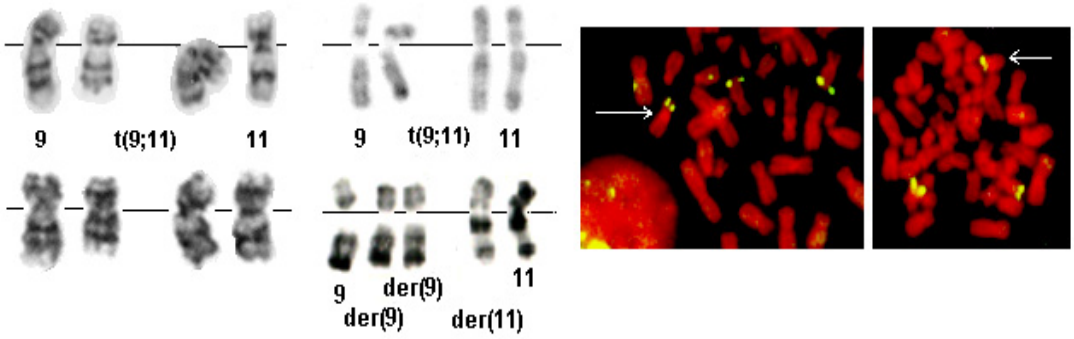

Figure 1-8 | Structure of the t(9;11)(p22;q23) translocation (Modified from www.atlasgeneticsoncology.org).

The translocation t(9;11)(p22;q23) results in the fusion of the portion of *MLL* gene at 11q23 to the portion of *AF9* gene (also called *MLL*T3) which maps to 9p22, giving rise to different *MLL-AF9* fusion genes (Figure 1-9) due to the involvement of different breakpoints of both genes. *MLL* breakpoints cluster in an 8.3 kb region that spans the portion of exon 8, exons 9 – 13 and the portion of exon 14, according to the most recent *MLL* exon numbering system (Nilson, Lochner et al. 1996). The most frequent *AF9* breakpoint cluster regions (BCRs) have been identified in a telomeric region of intron 4 (BCR1) and within introns 7 and 8 (BCR2) (Strissel, Strick et al. 2000).

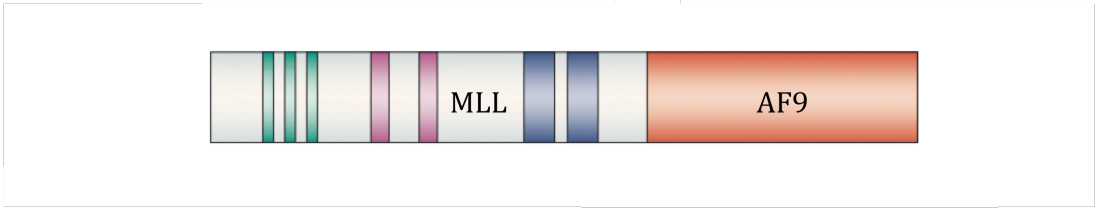

Figure 1-9 | Structure of the *MLL-AF9* fusion gene (Modified from Krivtsov and Armstrong 2007).

MLL-AF9-expressing AML cells have morphologic and immunophenotypic features consistent with myelomonocytic differentiation. Patients with an *MLL-AF9* fusion have an intermediate to poor prognosis, suggesting that *MLL-AF9* expression is associated with a more aggressive disease that includes resistance to chemotherapy (Schoch, Schnittger et al. 2003).

1.3.3. *FLT3-ITD* MUTATION IN ACUTE MYELOID LEUKEMIA

FLT3 is a cytokine receptor (Figure 1-10) that is expressed on the leukemic blasts in most cases of acute leukemia (Birg, Courcoul et al. 1992; Meierhoff, Dehmel et al. 1995; Carow, Levenstein et al. 1996; Drexler 1996). Upon binding FLT3 ligand (FL), FLT3 dimerizes and undergoes a conformational change, causing its activation loop to assume an open conformation and to allow ATP access to the ATP-binding pocket. Ligand-activated FLT3 undergoes autophosphorylation and, through a series of kinase cascades, transduces signals promoting cell growth and inhibiting apoptosis through proteins such as Ras-GTPase activating protein, phospholipase C, STAT5, and ERK1/2.6-12.

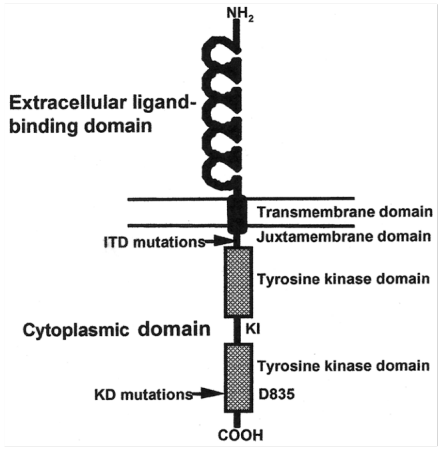

Figure 1-10 | Structure of the FLT3 receptor (Modified from Small 2006).

The ligand, FL, is expressed in virtually all cell types thus far examined, including leukemia cells (Lyman and Jacobsen 1998). In contrast, the receptor, FLT3, has a fairly narrow range of cell expression, being localized primarily to hematopoietic and neural tissues, which presumably confines its functions to these cell types (Small, Levenstein et al. 1994). FL acts in synergy with other cytokines to promote hematopoietic precursor expansion, and targeted disruption of either *FLT3* or *FL* in mice, although not embryonically lethal, leads to a reduction in hematopoietic precursors (Mackarehtschian, Hardin et al. 1995; Nicholls, Winter et al. 1999; McKenna, Stocking et al. 2000; Sitnicka, Buza-Vidas et al. 2003).

The *FLT3* gene has been found to be frequently mutated in AML cases, in the molecular form of Internal Tandem Duplications (*FLT3/ITD*). This mutation was first described in patients with AML in 1996 by Nakao et al. (Nakao, Yokota et al. 1996). FTL3/ITDs, which disrupt the autoinhibitory function of the receptor's juxtamembrane domain, result in constitutive autophosphorylation of FLT3 within the blasts that harbor them (Kiyoi, Towatari et al. 1998; Griffith, Black et al. 2004). Fifteen years after this initial discovery, *FLT3/ITD* AML now stands as a distinct clinical entity, an often lethal subtype of AML that has been a considerable challenge to those of us who treat it (Kindler, Lipka et al. 2010). Some recent clinical and laboratory findings about this disease may provide insight into why these patients relapse so quickly, and how would it be possible to improve their outcomes.

FLT3/ITD mutations are present in roughly a quarter of adult AML cases (Levis and Small 2003). In a minority of cases they represent a presumably late mutation in AML, evolving out of an antecedent myelodysplastic syndrome (Pinheiro, de Sa Moreira et al. 2008). However, the more characteristic presentation is that of *de novo* disease, presenting with a high leukocyte count and normal cytogenetics. Numerous retrospective analyses of clinical trial results have established that patients with *FLT3/ITD* AML achieve complete remission at or near the rate for patients with AML lacking these mutations (Kottaridis, Gale et al. 2001; Frohling, Schlenk et al. 2002; Schnittger, Schoch et al. 2002; Thiede, Steudel et al. 2002). However, equally well established is the fact that patients with *FLT3/ITD* are far more likely to relapse and do so more rapidly than their *FLT3* wild-type counterparts. The median survival of *FLT3* mutant AML after first relapse has been reported to be 5 months (Ravandi, Kantarjian et al. 2010; Levis, Ravandi et al. 2011).

FLT3 mutations are of clinical importance (Table 1-6). *FLT3* ITDs have been strongly associated with leukocytosis, high blast counts, normal cytogenetics, t(15;17) and t(6;9). Recently, one study indicated that *FLT3* ITDs might also be associated with

duplications and/or double-strand DNA breaks in the breakpoint cluster region of the *MLL* gene (Libura, Asnafi et al. 2003).

The prevalence of *FLT3* ITDs in patients with AML increases with age, ranging from 5–15% in pediatric patients to 25–35% in adults. Most studies in pediatric patients with AML have found that *FLT3* ITDs are strong, independent predictors of poor clinical outcome (Iwai, Yokota et al. 1999; Kondo, Horibe et al. 1999; Meshinchi, Woods et al. 2001) (Table 1-6).

Table 1-6 | Clinical relevance of *FLT3-ITD* mutations in patients with AML (Modified from Stirewalt and Radich 2003).

Number of patients in study	Frequency of *FLT3* ITDs (%)	Mean age of patients (years)	CR wild-type:ITD (%)	DFS wild-type:ITD (months)	OS wild-type:ITD (months)	5-year DFS wild-type:iTD (%)	5-year OS wild-type:ITD (%)
64	11	6	93:95	N.D.	N.D.	69:14	N.D.
94	5	6.8	N.D.	N.D.	N.D.	60:20	N.D.
91	17	10.5	74:40	N.D.	N.D.	N.D.	N.D.
201	23	N.D.	75:71 (N.S.)	N.D.	N.D.	50:18	45:14
979	20	N.D.	67:71 (N.S.)	18:13	14:10 (N.S.)	N.D.	N.D.
106	13	41	N.D.	N.D.	29:13	N.D.	N.D.
854	27	41	84:78	N.D.	N.D.	46:30	44:32
1003	23	57	70:70 (N.S.)	13:7	15:12 (N.S.)	N.D.	N.D.
140	27	67	40:55 (N.S.)	8:8 (N.S.)	7:8 (N.S.)	N.D.	N.D.

All results are statistically significant unless labeled as (N.S.). ML, acute myeloid leukemia; CR, complete response; DFS, disease-free survival; FLT3, FMS-like tyrosine kinase; ITD, internal tandem duplication; N.D., not determined; N.S., not statistically significant (P>0.05); OS, overall survival.

2. HEMATOPOIESIS

Hematopoiesis (from Ancient Greek: αἷμα, "blood"; ποιεῖν "to make") is the formation of blood cellular components. All cellular blood components are derived from hematopoietic stem cells. In a healthy adult person, approximately 10^{11}–10^{12} new blood cells are produced daily in order to maintain steady state levels in the peripheral circulation.

2.1. HEMATOPOIETIC HIERARCHY

Hematopoiesis is the process by which all the different cell lineages that form the blood and immune system are generated from a common pluripotent stem cell. During the life of an individual, two separate hematopoietic systems exist, both arising during embryonic development, but only one persisting in the adult. The primitive system is derived from the extraembryonic yolk sac and consists mainly of nucleated erythroid cells, which carry oxygen to the developing embryonic tissues. As the embryo increases in size, this early circulatory system is superseded by the more complex definitive system, which originates within the embryo itself and continues throughout adult life. This definitive hematopoietic system is made up of all adult blood cell types including erythrocytes and cells of the myeloid and lymphoid lineages. All these cells are derived from pluripotent hematopoietic stem cells (HSCs) through a succession of precursors with progressively limited potential under the control of specific cytokines such as interleukins and granulocyte/monocyte-stimulating factors. In most cases, the cytokines that determine differentiation to a particular lineage are well defined. The factors that regulate HSC generation and maintenance of pluripotency, however, remain largely unknown.

The first definitive multipotent HSCs are generated within the embryonic aorta-gonad-mesonephros (AGM) region (Figure 1-11) (www.rndsystems.com). The AGM extends from the umbilicus to the anterior limb bud of the human embryo and contains the dorsal aorta. Within the dorsal aorta, a cluster of $CD34^{+}$ hematopoietic cells is associated with the ventral floor of the aorta.

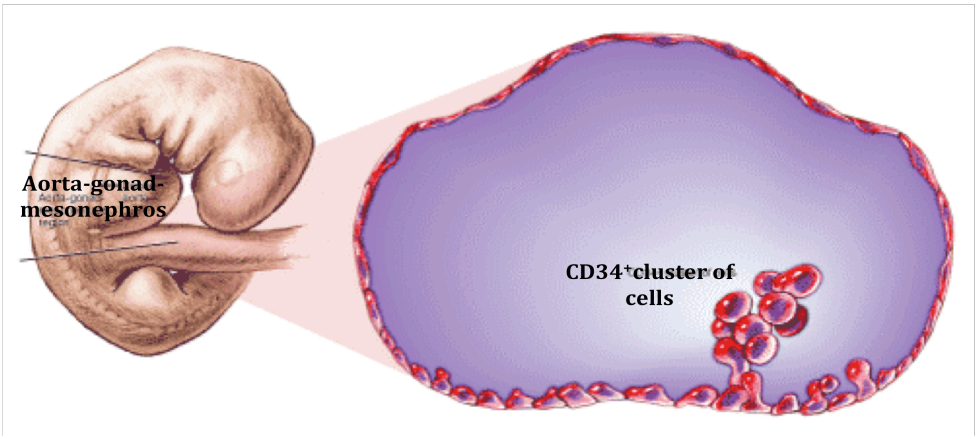

Figure 1-11 | Structure of the embryonic aorta-gonad-mesonephros (AGM) region (Modified from www.rndsystems.com).

The adult hematopoietic tissues (*i.e.* bone marrow, thymus and spleen) are seeded by multilineage blood cells derived from the fetal liver. During embryogenesis, however, fetal liver hematopoiesis is preceded by the emergence of pluripotent HSCs in a region of the para-aortic splanchnopleural mesoderm containing the dorsal aorta, gonadal ridge and mesonephros, named the aorta-gonad-mesonephros or AGM region (Figure 1-11). *In vitro* studies and repopulation analyses in myeloablated recipient mice have established this region as a major source of long-term repopulating (LTR)-HSCs between 8.5-11.5 days post coitum (dpc) in the mouse and 4-6 weeks gestation in the human embryo, prior to the onset of liver hematopoiesis (Medvinsky, Samoylina et al. 1993; Godin, Dieterlen-Lievre et al. 1995; Cumano, Dieterlen-Lievre et al. 1996; Medvinsky and Dzierzak 1996; Tavian, Coulombel et al. 1996; Tavian, Hallais et al. 1999) (Figure 1-12).

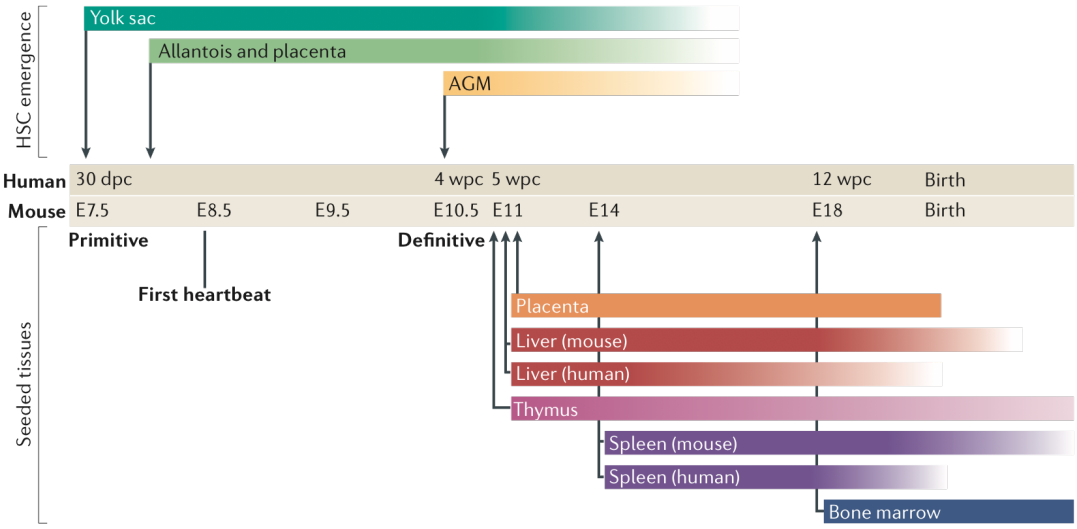

Figure 1-12 | Timeline of hematopoietic development in mice and humans (Modified from Wang and Wagers 2011).

Coincident with this period of LTR-HSC activity, clusters of rounded cells adhering to the ventral wall of the dorsal aorta and the umbilical and vitelline arteries (where they connect with the dorsal aorta) have been identified in both human and murine AGM regions *in vivo* (Tavian, Coulombel et al. 1996; Wood, May et al. 1997). Expression analysis of these cell clusters reveals that they express the hematopoietic-specific marker CD45 as well as a number of markers in common with adjacent endothelial cells including the membrane glycoprotein CD34, which is commonly used to identify HSCs in bone marrow and peripheral blood (Marshall and Thrasher 2001). CD34-positive cells isolated from murine AGM at 10.5 dpc can give rise to cells of all hematopoietic lineages *in vitro* (Delassus, Titley et al. 1999). It is now widely accepted that these intra-aortic cell clusters constitute the first site of definitive HSC generation during development and represent the origins of adult hematopoiesis.

The source of these first HSCs and the mechanisms by which their tightly regulated appearance and subsequent disappearance are controlled are currently under investigation. The shared expression patterns of a number of molecules by both intra-aortic cluster cells and underlying endothelial cells supports the existence of a hemangioblast or endothelial-like cell with hemogenic potential that resides within the ventral floor of the dorsal aorta (Marshall and Thrasher 2001) (Figure 1-13).

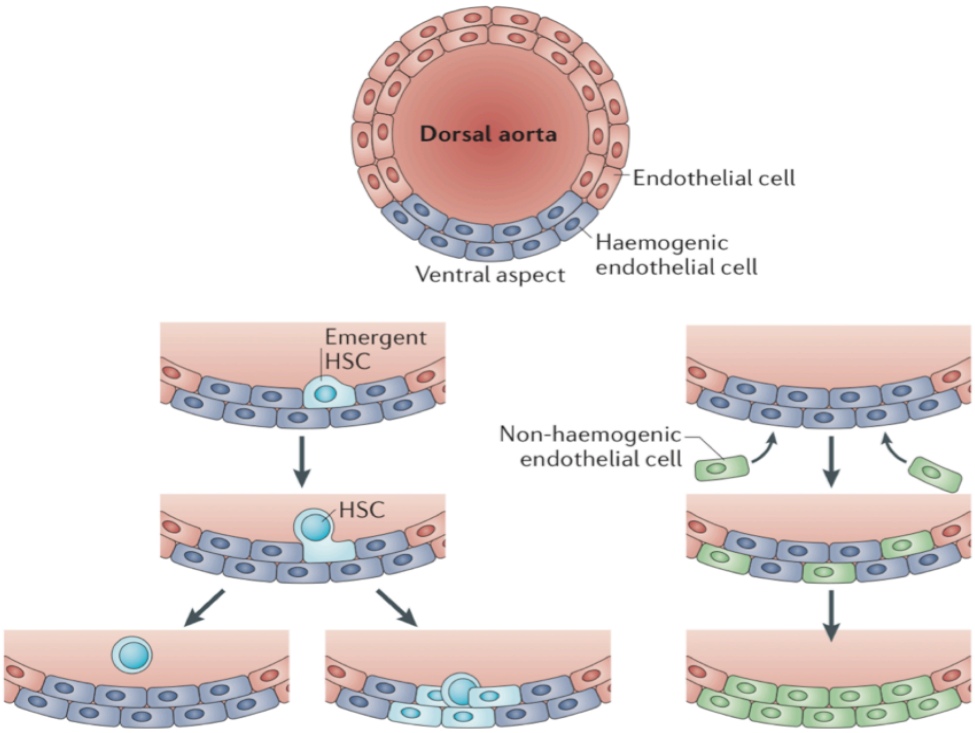

Figure 1-13 | Definitive fetal hematopoiesis in the AGM (Modified from Wang and Wagers 2011).

Within the embryonic AGM region, the eventual fate of these hemangioblasts would be determined by factors that bind at the cell surface, triggering downstream signaling pathways that culminate in the activation of hematopoietic or other lineage-specific genes. The identification of a morphologically distinct region of cells, resembling a stromal layer, underlying the ventral floor of the dorsal aorta within the AGM has suggested that this region could represent a microenvironment, or niche, supporting HSC development (Marshall and Thrasher 2001). This highly defined ventral region, coupled with the absence of contaminating committed hematopoietic progenitors within the AGM at this stage of development, provides an ideal environment in which to investigate the factors involved in the generation and regulation of HSCs *in vivo*.

2.2. HEMATOPOIETIC STEM CELLS (HSCs)

The first experimental evidence to indicate the existence of HSCs was the discovery in 1961 by Till and McCulloch (Till and Mc 1961) of a population of clonogenic bone marrow cells capable of generating myelo-erythroid colonies in the spleen of lethally irradiated hosts. Occasionally these colonies contained clonogenic cells that could be further retransplanted into secondary lethally irradiated hosts and

reconstitute the immune system. These were proposed to be HSCs, i.e., progenitor cells with the essential characteristic of self-renewal and differentiation potential for all types of blood cells (Spangrude, Heimfeld et al. 1988; Morrison and Weissman 1994; Osawa, Hanada et al. 1996). The development of clonal assays for all major hematopoietic lineages together with the availability of multiparameter fluorescence-activated cell sorting (FACS) has enabled the prospective purification of HSCs from mice and to highly enrich for HSCs from humans according to the cell- surface expression of specific molecules and their functional read-out in vivo and in vitro in stromal long-term colony- initiating assays (Domen and Weissman 1999). After the identification and prospective isolation of murine HSCs, considerable progress has been made toward the characterization of the mechanisms controlling their fates. During or after cell division, the two daughter cells of a stem cell each have to decide their fate. They can either choose to remain as HSCs, commit to differentiation, or die by apoptosis and also to stay in the bone marrow or migrate to the periphery. These processes of cell-fate decisions must be finely tuned to maintain a steady-state level of functional HSCs in the bone marrow and to constantly provide progenitors for the various hematopoietic lineages.

Hematopoietic development (Figure 1-14) is initiated from long-term hematopoietic stem cells (LT-HSCs) that are multipotent and have the potential to self-renew (Spangrude, Heimfeld et al. 1988). In the adult bone marrow, LT-HSCs differentiate into multipotent progenitor cells (MPPs). The initial view was that MPPs are precursors for the two major lineage- restricted progenitors: a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP) population (Kondo, Weissman et al. 1997; Akashi, Traver et al. 2000). Progeny derived from CLPs segregate into either the B-cell or the T-cell lineage (Kondo, Weissman et al. 1997). The CMPs give rise to either the granulocyte/macrophage progenitors (GMP) or megakaryocytic/erythrocyte progenitors (MEPs) (Akashi, Traver et al. 2000).

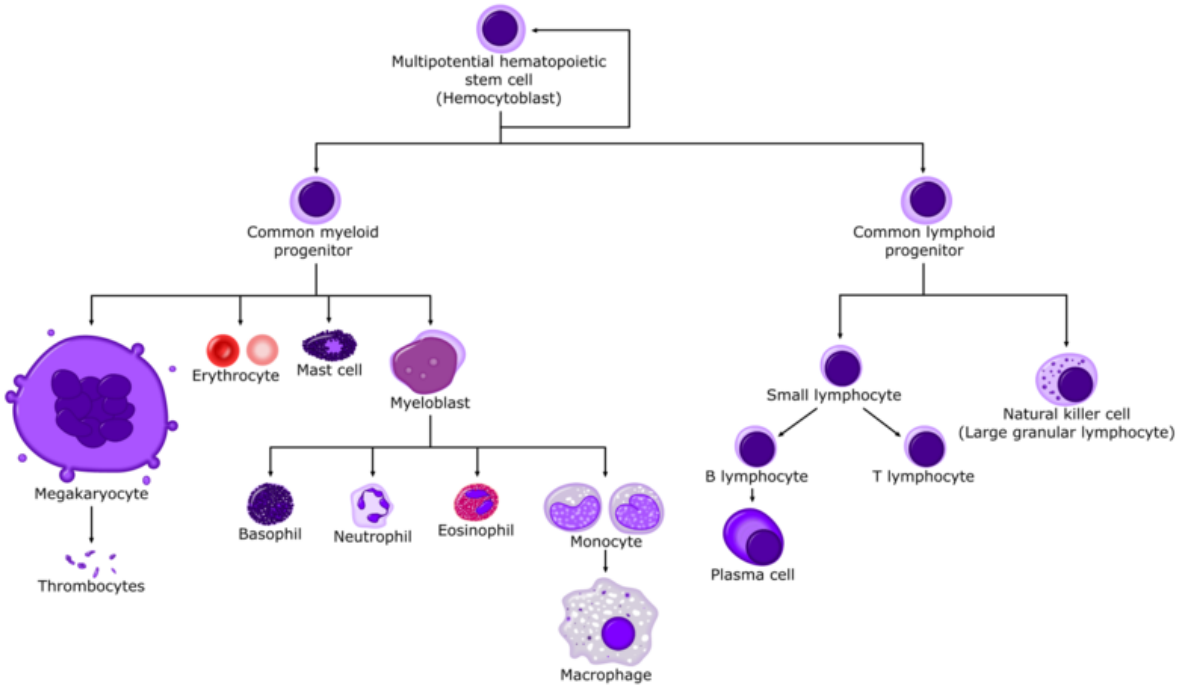

Figure 1-14 | Schematic representation of the hematopoietic development (Modified from en.wikipedia.org).

HSCs have a number of unique properties, the combination of which defines them as such (Domen and Weissman 1999). Among the core properties are the ability to choose between self-renewal (remain a stem cell after cell division) or differentiation (start the path towards becoming a mature hematopoietic cell). In addition, HSCs migrate in regulated fashion and are subject to regulation by apoptosis (programmed cell death). The balance between these activities determines the number of stem cells that are present in the body.

One essential feature of HSCs is the ability to self-renew, that is, to make copies with the same or very similar potential. This is an essential property because more differentiated cells, such as hematopoietic progenitors, cannot do this, even though most progenitors can expand significantly during a limited period of time after being generated. However, for continued production of the many (and often short- lived) mature blood cells, the continued presence of stem cells is essential. While it has not been established that adult HSCs can self-renew indefinitely (this would be difficult to prove experimentally), it is clear from serial transplantation experiments that they can produce enough cells to last several (at least four to five) lifetimes in mice. It is still unclear which key signals allow self-renewal. One link that has been noted is telomerase, the enzyme necessary for maintaining telomeres, the DNA regions at the end of chromosomes that protect them from accumulating damage due to DNA

replication. Expression of telomerase is associated with self-renewal activity. However, while absence of telomerase reduces the self-renewal capacity of mouse HSCs, forced expression is not sufficient to enable HSCs to be transplanted indefinitely; other barriers must exist (Allsopp, Morin et al. 2003; Allsopp, Morin et al. 2003).

Differentiation into progenitors and mature cells that fulfill the functions performed by the hematopoietic system is not a unique HSC property, but, together with the option to self-renew, defines the core function of HSCs. Differentiation is driven and guided by an intricate network of growth factors and cytokines. As discussed earlier, differentiation, rather than self- renewal, seems to be the default outcome for HSCs when stimulated by many of the factors to which they have been shown to respond. It appears that, once they commit to differentiation, HSCs cannot revert to a self-renewing state. Thus, specific signals, provided by specific factors, seem to be needed to maintain HSCs. This strict regulation may reflect the proliferative potential present in HSCs, deregulation of which could easily result in malignant diseases such as leukemia or lymphoma.

Migration of HSCs occurs at specific times during development (i.e., seeding of fetal liver, spleen and eventually, bone marrow) and under certain conditions (e.g., cytokine-induced mobilization) later in life. The latter has proven clinically useful as a strategy to enhance normal HSC proliferation and migration, and the optimal mobilization regimen for HSCs currently used in the clinic is to treat the stem cell donor with a drug such as cytoxan, which kills most of his or her dividing cells. Normally, only about 8% of LT-HSCs enter the cell cycle per day (Bradford, Williams et al. 1997; Cheshier, Morrison et al. 1999), so HSCs are not significantly affected by a short treatment with cytoxan. However, most of the downstream blood progenitors are actively dividing (Kondo, Weissman et al. 1997; Akashi, Traver et al. 2000), and their numbers are therefore greatly depleted by this dose, creating a demand for a regenerated blood-forming system. Empirically, cytokines or growth factors such as G-CSF and KitL can increase the number of HSCs in the blood, especially if administered for several days following a cytoxan pulse. The optimized protocol of cytoxan plus G-CSF results in several self-renewing cell divisions for each resident LT-HSC in mouse bone marrow, expanding the number of HSCs 12- to 15-fold within two to three days (Morrison, Wright et al. 1997). Then, up to one-half of the daughter cells of self-renewing dividing LT-HSCs (estimated to be up to 105 per mouse per day (Wright, Wagers et al. 2001)) leave the bone marrow, enter the blood, and within minutes engraft other hematopoietic sites, including bone marrow, spleen, and liver (Wright, Wagers et al. 2001). These migrating cells can and do enter empty hematopoietic niches elsewhere in the bone marrow and provide sustained hematopoietic stem cell

self-renewal and hematopoiesis (Wright, Wagers et al. 2001; Abkowitz, Robinson et al. 2003). It is assumed that this property of mobilization of HSCs is highly conserved in evolution (it has been shown in mouse, dog and humans) and presumably results from contact with natural cell-killing agents in the environment, after which regeneration of hematopoiesis requires restoring empty HSC niches. This means that functional, transplantable HSCs course through every tissue of the body in large numbers every day in normal individuals.

2.3. NORMAL AND LEUKEMIC HEMATOPOIESIS

Leukemia can be viewed as a newly formed, abnormal hematopoietic tissue initiated by a few leukemic stem cells (LSCs) that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal hematopoietic stem cells. A hallmark of all cancers is the capacity for unlimited self-renewal, which is also a defining characteristic of normal stem cells. Given this shared attribute, it has been proposed that leukemias may be initiated by transforming events that take place in hematopoietic stem cells. Alternatively, leukemias may also arise from more committed progenitors caused by mutations and/or selective expression of genes that enhance their otherwise limited self-renewal capabilities.

In case of normal hematopoiesis (Figure 1-15) we talk about a process involving normal and healthy cells, beginning from ES cells, through hematopoietic stem cells and multipotent progenitors, that possesses the ability of self renewal and proliferation and can further differentiate into lymphoid or myeloid progenitors, giving rise to mature blood cells (lymphocytes, erythrocytes, etc.). During the normal hematopoiesis, there has to be a tight control between these two driving forces that co-exist in HSCs; there should be a balance between proliferation and differentiation.

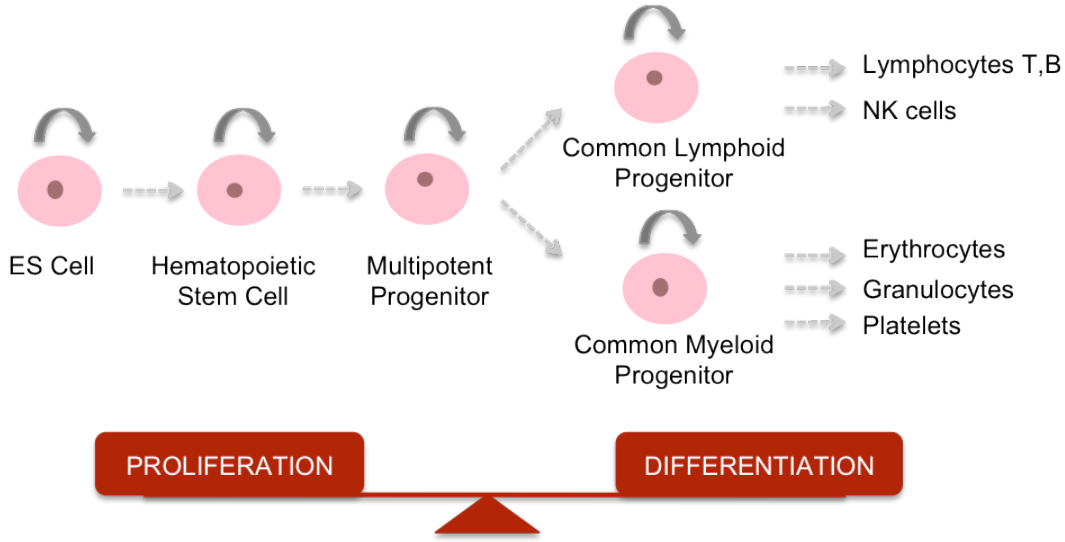

Figure 1-15 | Schematic representation of normal hematopoiesis.

However, this normal process of hematopoiesis can be altered, for example by the presence of some genetic events (such as mutations or chromosome translocations) that target essential genes. It is known from the literature that these genetic events can occur at different levels of differentiation, leading to leukemogenesis (Figure 1-16). The consequence of such an oncogenic event will be the accumulation of immature/malignant progenitor cells, an increased proliferation and a blocked differentiation that, at the end, will lead to leukemia development.

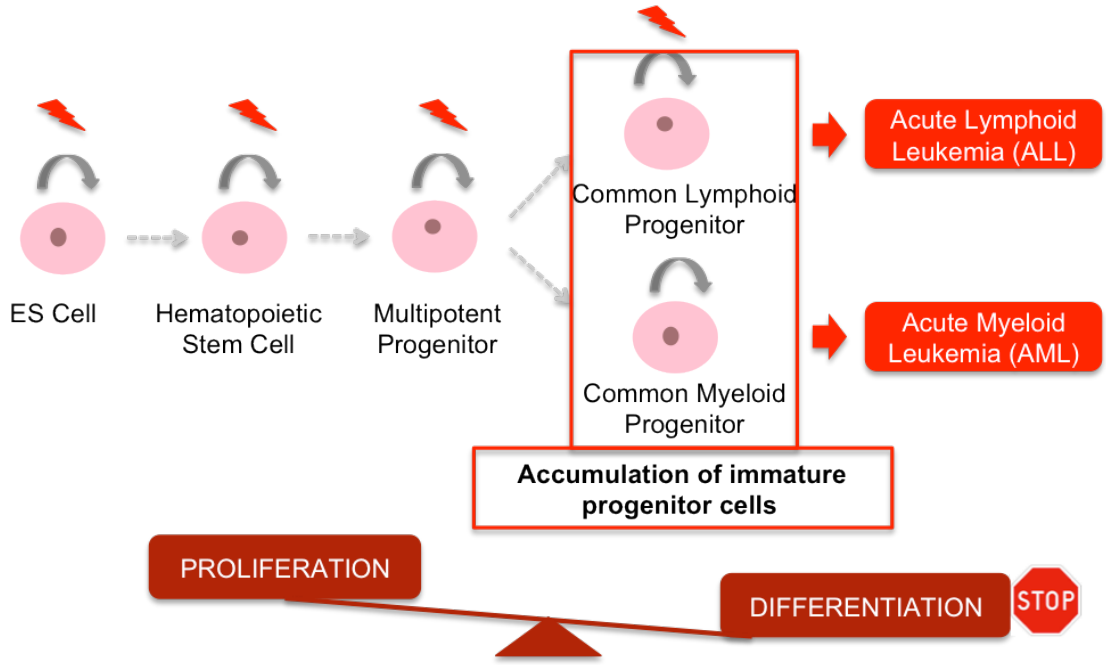

Figure 1-16 | Schematic representation of leukemic hematopoiesis.

3. HUMAN EMBRYONIC STEM CELLS AS A TOOL TO MODEL BLOOD DISEASES

It is 15 years since the first report of human embryonic stem (hES) cells. The enormous potential of these cells for medical applications was almost immediately clear: they might provide unlimited supplies of a wide variety of cell types for use in new therapies, drug screening, toxicology and disease modeling. The major hurdle to the development of embryonic stem cell (ES cell) applications comes precisely from the property that defines their great potential: their propensity to differentiate into specialized cell types of the body. To use this property in medical applications, it is crucial that we understand how to control the cells – both their proliferation (or multiplication) as undifferentiated stem cells, and their differentiation into particular specialized cell types among the myriad possibilities (Huber 2010).

Isolated from the preimplantation blastocyst approximately 5 to 7 days following in vitro fertilization (IVF), hES cells can differentiate into representatives of all 3 embryonic germ layers (endoderm, mesoderm, and ectoderm) as well as cells with properties of extraembryonic trophectoderm (Xu, Chen et al. 2002; Gerami-Naini, Dovzhenko et al. 2004; Zaehres, Lensch et al. 2005). Methodologies allowing for directed differentiation of hES cells have been reported for a variety of key cellular types, including cardiac myocytes (Kehat, Khimovich et al. 2004), neural progenitors (Reubinoff, Pera et al. 2000; Reubinoff, Itsykson et al. 2001; Ben-Hur, Idelson et al. 2004), and hematopoietic tissue (Kaufman, Hanson et al. 2001). Early experiments with hES cells have lacked the experimental wizardry of contemporary murine studies due to the dearth of key platform technologies. This is changing rapidly given reports demonstrating genetic modification of hES cells using retro/lentiviral transgenesis (Gropp, Itsykson et al. 2003; Ma, Ramezani et al. 2003; Zaehres, Lensch et al. 2005), homologous recombination (Zwaka and Thomson 2003), and RNA interference (RNAi) (Hay, Sutherland et al. 2004; Matin, Walsh et al. 2004; Vallier, Rugg-Gunn et al. 2004; Zaehres, Lensch et al. 2005). An experimental target gene has not yet been reported that is comparable to the ROSA-26 locus (Zambrowicz, Imamoto et al. 1997) in mice, a region of chromatin permissive for the constitutive expression of transgenes and useful for expressing factors such as transactivators in conditional gene expression systems (Gossen and Bujard 1992). However, gene-trapping studies are under way in hES cells (Dhara and Benvenisty 2004).

Despite the need to develop basic technologies that enable their manipulation, hES cells have already proven quite useful in the study of hematopoiesis. In a cohort of manuscripts, Bhatia's group has reported serum-based cultivation conditions with

mesoderm/ hematopoietic cell-inducing cytokines (BMP-4, VEGF165, IL-3, IL-6, SCF, FLT-3L, and G-CSF) that lead to a high percentage of blood cells in culture (Murdoch, Gallacher et al. 2002; Chadwick, Wang et al. 2003; Cerdan, Rouleau et al. 2004; Menendez, Wang et al. 2004; Wang, Li et al. 2004; Wang, Li et al. 2005; Wang, Menendez et al. 2005). However, this hematopoietic tissue is limited in its capacity to engraft in animal transplantation models due in part to an abnormal expression profile for *HOX* A- and B-cluster genes (Wang, Menendez et al. 2005). It has been have also studied the elaboration of lympho-myeloid hematopoietic tissue from hES cells via different culture methodologies (Lu, Li et al. 2004; Tian, Morris et al. 2004; Zhan, Dravid et al. 2004; Ng, Davis et al. 2005; Vodyanik, Bork et al. 2005; Zambidis, Peault et al. 2005), although again, no hematopoietic engraftment has been demonstrated. What is clear, however, is that a trajectory of discovery similar to that seen in comparable murine studies is present in the hES cell research, with an arc that points to engraftable hematopoietic cells likely being generated in the next few years. The present inability to demonstrate engraftment aside, hES cell-based studies are likely to prove particularly valuable for research aimed at understanding genetic diseases of the blood (Table 1-7).

Human embryonic stem cells (hESC) are envisioned to become a powerful tool for modeling different aspects of human diseases that cannot otherwise be addressed by patient sample analyses or mouse models (Thomson, Itskovitz-Eldor et al. 1998; Menendez, Bueno et al. 2005). The fact that leukemogenesis manifests as altered cell differentiation suggests that hematopoietic-directed differentiation of hESCs could become a promising human- specific strategy to study the onset of hematopoiesis, particularly the emergence of the earliest events leading to the specification of both normal and abnormal hematopoietic tissue (Lensch and Daley 2006; Bueno, Montes et al. 2012).

Table 1-7 | Congenital hematologic diseases lending themselves to study using hESCs (Modified from Lensch and Daley 2006).

Disease	OMIM*	Genes	Blood phenotype
Fanconi anemia	227650	Several including BRCA2	Aplastic anemia, pancytopenia, MDS, AML
TAR syndrome	274000	Unknown	Pancytopenia, anemia, hypercelular marrow, granulocytosis
Severe congenital neutropenia	202700	Neutrophils elastase	Myeloid arrest, AML, agranulocytosis
Schwachman-Diamond syndrome	260400	SBDS	Anemia, thrombocytopenia, pancytopenia, MDS, AML
Diamond-Blackfan anemia	205900	Ribosomal protein S19, unknown	Macrocytic anemia, thrombocytopenia, fetal hemoglobin
Trisomy 21	190685	N/A	Transient myeloproliferative disease, DS-AMKL
Chuvash polycythemia	263400	VHL	Erythrocytosis, increased erythropoietin
Neurofibromatosis type 1	162200	Neurofibromin	JMML
Lesch-Nyhan syndrome	300322	HPRT1	Megaloblastic anemia
SCID	601457	Several including ADA, RAG1/2	Lymphopenia, lack of mature B and T cells
α-thalassemia	141800	α-globin	Methemoglobina, polycythemia, hemolysis
β-thalassemia	14100	β-globin	Anemia, hypochromic microcytes, splenomegaly
Sickle cell anemia	603903	β-globin	Anemia, septicemia, microvascular occlusion

TAR indicates thrombocytopenia-absent radius; SCID, severe immunodeficiency; BRCA2, breast cancer type 2 gene; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; SBDS, Schwachman-Bodie-Diamond syndrome gene; DS-AMKL, Down syndrome-associated acute megakaryoblastic leukemia; VHL, von Hippel-Lindau gene; JMML, juvenile myelomonocytic leukemia; HPRT, hypoxanthine guanine phosphoribosyltransferase 1 gene, ADA, adenosine deaminase; RAG, recombination activating gene.
*Online Mendelian Inheritance in Man, OMIM. World Wild Web URL: http://www.ncbi.nlm.nih.gov/omim/.

OBJECTIVES

Leukemias harboring the *MLL-AF9* fusion gene are associated with a dismal prognosis and their clinical consequences are well characterized. Concerning the biological processes affected by this fusion gene, little is known and our understanding of its transformation capacities is still limited. For instance, it is not known at which stage of hematopoietic differentiation this translocation may occur and if there, in fact, is a critical one in the process of leukemogenesis. Accordingly, this study is aimed to the following objectives:

1. Develop a cellular model of the leukemic fusion gene *MLL-AF9* in human Embryonic Stem Cells.

2. Characterize the biology of the model in terms of its differentiation pattern, its chromosomic stability and its proliferative parameters.

3. Address the following questions:

- Which is the effect of the expression of the leukemogenic *MLL-AF9* fusion gene on the process of hematopoiesis-directed differentiation from human Embryonic Stem Cells?
- Which is the effect of an additional leukemogenic hit, such as the *FLT3* Internal Tandem Duplication, on the process of hematopoiesis-directed differentiation from human Embryonic Stem Cells?

OBJETIVOS

Las leucemias caracterizadas por la presencia del gen de fusión *MLL-AF9* están asociadas con un mal pronóstico y sus consecuencias clínicas están bien caracterizadas. Por el contrario, nuestro conocimiento tanto sobre los procesos biológicos afectados por este gen de fusión como sobre sus capacidades de transformación es limitado. Por ejemplo, no se sabe con certeza en qué etapa o etapas de la diferenciación hematopoyética puede aparecer esta translocación y cuál es, si la hay, una etapa critica para el proceso de desarrollo de la leucemia. Por consiguiente, este estudio se dirige a los siguientes objetivos:

1. Desarrollar un modelo celular del gen leucemogénico de fusión *MLL-AF9* en células troncales humanas de naturaleza embrionaria.

2. Caracterizar la biología del modelo en términos de su patrón de diferenciación, su estabilidad cromosómica y sus parámetros proliferativos.

3. Intentar responder a las siguientes preguntas:

    - ¿Cuál es el efecto de la expresión del gen leucemogénico de fusión *MLL-AF9* en el proceso de la diferenciación hematopoyética de las células troncales humanas de naturaleza embrionaria?

    - ¿Cuál es el efecto de un adicional golpe leucemogénico, como la *FLT3* Duplicación Interna en Tándem (*FLT3-ITD*), sobre el proceso de la diferenciación hematopoyética dirigida a partir de células troncales humanas de naturaleza embrionaria?

MATERIAL AND METHODS

3.1. MATHERIAL

3.1.1. EQUIPMENT

Autoclave	Technomara, Germany
Centrifuges	Heraeus, Hanau; Eppendorf, Germany
Electrophoresis equipment	Biometra, Göttingen; BioRad, Germany
FACS Fortessa, Calibur	BD, Heidelberg, Germany
Heatblock	Eppendorf, Germany
Incubators	Heraeus, Germany
J6M-E centrifuge	Beckman, Germany
Laminar air flow cabinet	Heraeus, Germany
Multipipettor Multipette plus	Eppendorf, Germany
NanoDrop	Thermo Fisher Scientifci, USA
PCR-Thermocycler PTC-200	Eppendorf, Germany
PCR-Thermocycler Veriti 384 well	Applied Biosystem, USA
pH-Meter	Eppendorf, Germany
Sigma 2 – Sartorius	Sartorius, Germany
Sorvall RC 6 plus	Thermo Fisher Scientific, Germany
Thermomixer	Eppendorf, Germany
Ultracentrifuge Optima L-70	Beckman, Germany
Waterbath	Julabo, Germany

3.1.2. CONSUMABLES

384-well PCR plates	Thermo Fisher Scientific, USA
8-channel pipettor tips Impact 384	Thermo Fisher Scientific, USA
Adhesive PCR sealing film	Thermo Fisher Scientific, USA
Cell culture flasks and plates	BD Falcon, USA; Corning, USA
Cell scraper	BD Falcon, USA
Cryo-tubes	BD Falcon, USA
FACS Filcon filters	Millipore, USA
PCR plate Twin.tec 96 well	Applied Biosystems, USA
Petri plates	BD Falcon, USA
Sterile combitips for Eppendorf multipette	Millipore, USA
Sterile plastic pipettes	BD Falcon, USA
Steritop GP Filter	Millipore, USA
Streacking Sticks “L”	VWR, Spain
Syringes and needles	BD Falcon, USA
Tubes for bacterial culture	BD Falcon, USA

3.1.3. CHEMICALS

All reagents used were purchased from Sigma-Aldrich (Germany), Gibco (Invitrogen, USA) or StemCell Technologies (Canada) unless otherwise noted. Oligonucleotides for Real-Time PCR were synthesized and high-pressure liquid chromatography purified by TaqMan (Applied Biosystems, USA). Oligonucleotides designed for qualitative PCR were purchased from Sigma-Aldrich (Spain) and Invitrogen (Spain).

3.1.4. ENZYMES AND KITS

Activin A	Sigma-Aldrich, Germany
1-Thioglycerol	Sigma-Aldrich, Germany
Blood & Cell Culture DNA Midi Kit	Qiagen, Germany
$CD34^+$ Separation Kit	StemCell Technologies, Canada
Collagenase IV	Gibco, Invitrogen, USA
Collagenzase B	Gibco, Invitrogen, USA
DAB Liquid + Substract	Dako, USA
Dispase	StemCell Technologies, Canada
DNA Ladder 1 kb plus	NEB, Great Britain
DNA molecular weight standard	Invitrogen, USA
DNAeasy Tissue & Blood Kit	Qiagen, Germany
dNTPs	Roche, Germany
Gel Extraction Kit	Qiagen, Germany
High Capacity cDNA Reverse Transcriptase Kit	Applied Biosystems, USA
Matrigel	Beckton Dickinson, USA
MEM Non-essential Aminoamcids	Gibco, Invitrogen, USA
MethoCult H4230	StemCell Technologies, Canada
Paraformaldehyde	Sigma-Aldrich, Germany
Plasmid Maxi Kit	Qiagen, Germany
Plasmid Mini Kit	Qiagen, Germany
Prolonged Antiphase DAPI	Invitrogen, USA
RNAsin RNA Inhibitor	Promega, USA
Spin Miniprep Kit	Qiagen, Germany
StemPro Accutase	StemCell Technologies, Canada
SyberGreen	Applied Biosystems, USA
TagPCR Reaction Mix	Applied Biosystems, USA
TaqDNA polymerase	Invitrogen, USA
TrypleSelect	Gibco, Invitrogen, USA
β-Mercaptoethanol	Gibco, Invitrogen, USA

3.1.5. CYTOKINES

bFGF	IBIAN Technologies, Spain
EPO	Peprotech, USA
FLT-3	Peprotech, USA
GM-CSF	Peprotech, USA
hBMP-4	Peprotech, USA
hSCF	Peprotech, USA
IL-3	Peprotech, USA
IL-6	Peprotech, USA
TPO	Peprotech, USA

3.1.6. OLIGONUCLEOTIDES

Pluripotency of hESCs

NANOG	Forward: 5’ TGCAGTTCCAGCCAAATTCTC 3’
	Reverse: 5’ CCTAGTGGTCTGCTGTATTACATTAAGG 3’
OCT3/4	Forward: 5’ TCTGCAGAAAGAACTCGAGCAA 3’
	Reverse: 5’ AGATGGTCGTTTGGCTGAACAC 3’
SOX2	Forward: 5’ CCCCCGGCGGCAATAGCA 3’
	Reverse: 5’ TCGGCGCCGGGGAGATACAT 3’
REX1	Forward: 5’ CAGATCCTAAACAGCTCGCAGAAT 3’
	Reverse: 5’ GCGTACGCAAATTAAAGTCCAGA 3’

The integration of the *MLL-AF9*

MLL-AF9	Forward: 5' CAGAGCAAACAGAAAAAAGTG 3' Reverse: 5' CTATAAGGTTCACGATCTGC 3'

The expression of *MLL-AF9*

MLL-AF9	Hs03296417_ft TaqMan Gene Expression Assay

The integration of the *FLT3-WT* and the *FLT3-ITD*

FLT3 endogenic	Forward: 5' GCAATTTAGGTATGAAAGCCAGC 3' Reverse: 5' GGTTGCCGTCAAAATGCTGAAAG 3'
FLT3-WT cDNA	Forward: 5' TGTCGAGCAGTACTCTAAACA 3' Reverse: 5' GAGTTTGGGAAGGTACTAGGAT 3'
FLT3-ITD cDNA	Forward: 5' GCAATTTAGGTATGAAAGCCAGC 3' Reverse: 5' GGTTGCCGTCAAAATGCTGAAAG 3'

The expression of *FLT3*

FLT3	Forward: 5' TGGAATTTCTGGAATTTAAGTCG 3' Reverse: 5' CTTGTCACCCACGGGAAA 3'

The expression of *MLL-AF9* target genes

HOXA9	Forward: 5' AAAACAATGCTGAGAATGAGAGC 3' Reverse: 5' TATAGGGGCACCGCTTTTT 3'
HOXB3	Forward: 5' AAAGAGTCGAGGCAAACGTC 3' Reverse: 5' TGGAGCTGGAGAAGGAGTTC 3'
HOXB4	Forward: 5' CTGGATGCGCAAAGTTCAC 3' Reverse: 5' AGCGGTTGTAGTGAAATTCCTT 3'
MEIS1	Forward: 5' GCATGAATATGGGCATGGA 3' Reverse: 5' CATACTCCCCTGGCATACTTTG 3'
MLL	Forward: 5' GGCCTGAATTTCTCCACAGA 3' Reverse: 5' TTCGACAGACGCTGTAGGTG 3'

3.1.7. ANTIBIOTICS

G418 (Neomycine)	Sigma-Aldrich, Germany
Ampicilline	Sigma-Aldrich, Germany
Penicillin-Streptomycin	Gibco, Invitrogen, USA

3.1.8. PLASMIDS

All lentiviral vectors were kindly provided by Dr. Pablo Menéndez (Genyo, Granada, Spain). The following vectors were used in this study:

pRRL-EF1α-PGK-NEO (control, NEO)
pRRL-EF1α-MLL-AF9-PGK-NEO (MLL-AF9)
pRRL-EF1α-FLT3-WT-PGK-EGFP (FLT3-WT)
pRRL-EF1α-FLT3-ITD-PGK-EGFP (FLT3-ITD)

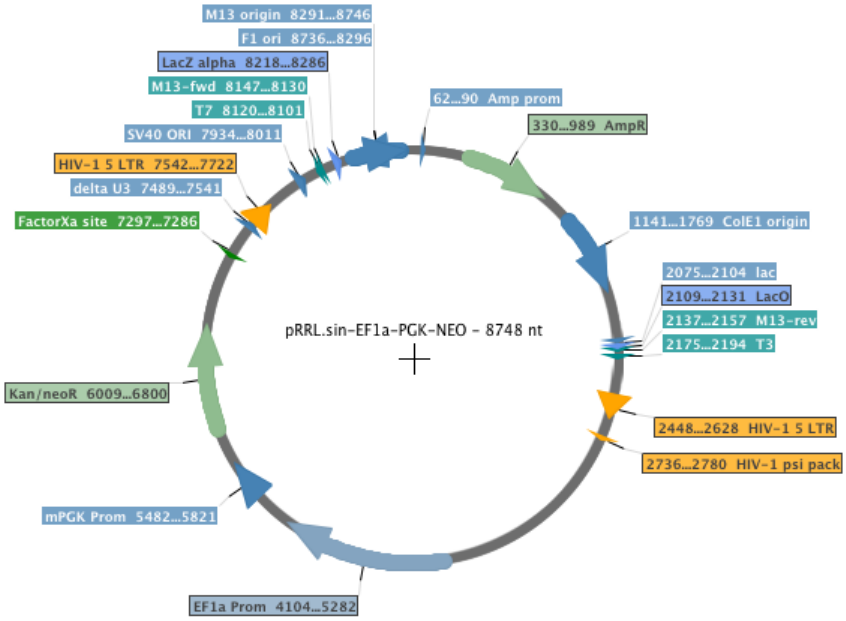

Figure 3-1 | Circular map of the MLL-NEO lentiviral vector.

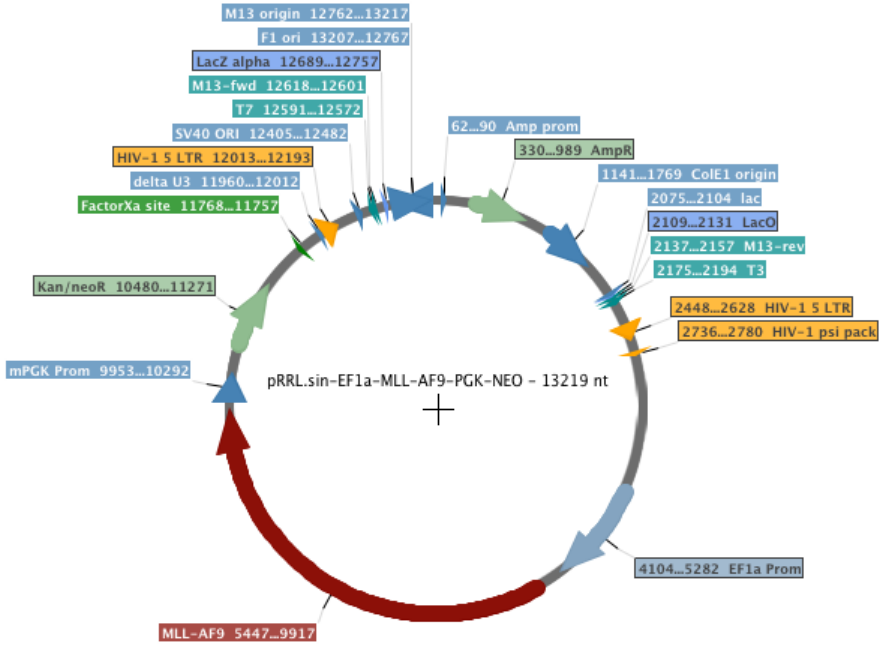

Figure 3-2 | Circular map of the MLL-AF9 lentiviral vector.

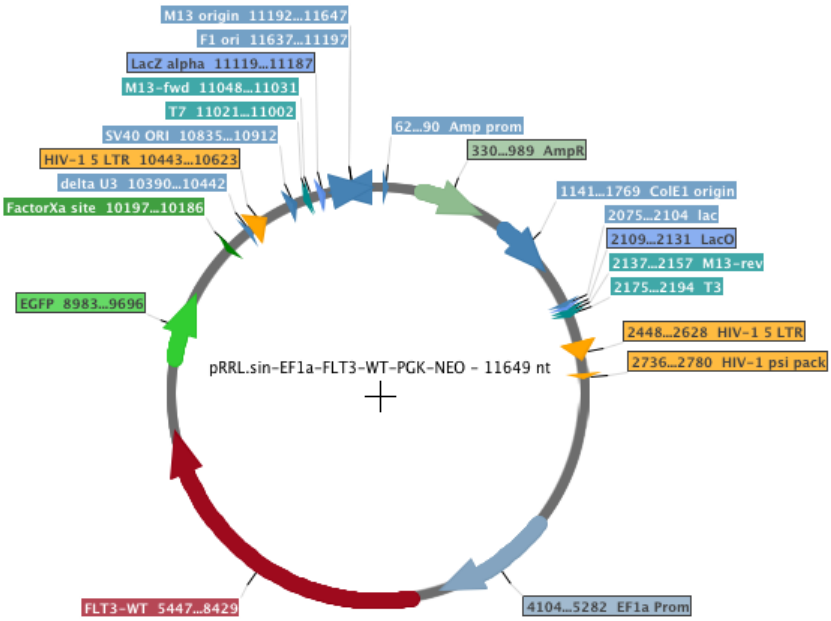

Figure 3-3 | Circular map of the FLT-WT lentiviral vector.

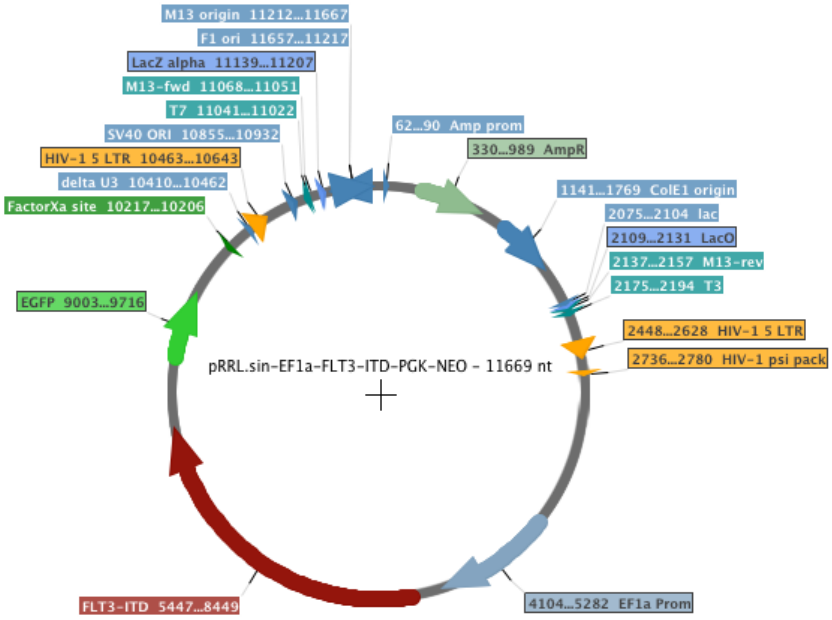

Figure 3-4 | Circular map of the FLT3-ITD lentiviral vector.

3.1.9. *E.COLI* STRAINS

DH10B	Invitrogen, USA
Stbl2	Invitrogen, USA

3.1.10. ANTIBODIES

FACS staining (Hematopoietic Differentiation)

CD31-FITC mouse anti-human	BD Pharmingen, USA
CD31-PE mouse anti-human	BD Pharmingen, USA
CD34-APC mouse anti-human	BD Pharmingen, USA
CD34-PE mouse anti-human	BD Pharmingen, USA
CD34-PerCP* mouse anti-human	BD Pharmingen, USA
CD34-PeCy7* mouse anti-human	BD Pharmingen, USA
CD45-APC mouse anti-human	BD Pharmingen, USA
CD45-PE mouse anti-human	BD Pharmingen, USA

Immunofluorescence (Pluripotency state of hESCs)

SSEA3 rat anti-human	Abcam, USA
SSEA4 mouse anti-human	Abcam, USA
TRA-1-60 mouse anti-human	Abcam, USA
TRA-1-81 mouse anti-human	Abcam, USA
OCT3/4 goat anti-human	Abcam, USA
TRA-1-60-Alexa Fluor 647 mouse anti-human	BD Pharmingen, USA
TRA-1-81-Alexa Fluor 647 mouse anti-human	BD Pharmingen, USA
Alexa Fluor 488 secondary goat anti-mouse IgG	Invitrogen, USA
Alexa Fluor 488 secondary donkey anti-goat IgG	Invitrogen, USA
Alexa Fluor 647 secondary goat anti-mouse IgG	Invitrogen, USA
Alexa Fluor 647 secondary goat anti-mouse IgM	Invitrogen, USA
Alexa Fluor 647 secondary donkey anti-goat IgG	Invitrogen, USA
Alexa Fluor 647 secondary goat anti-rat	Invitrogen, USA
HRP goat anti-rat	Invitrogen, USA

3.1.11. CELL LINES

AND2 Human Embryonic Cell Line	Andalusian Stem Cell Bank, Granada, Spain
hMSC Human Mesenchymal Stem Cell Line	National Cell Line Bank, Madrid, Spain
293T Human Embryonic Kiney Cell Line	National Cell Line Bank, Madrid, Spain

3.1.12. DATABASES AND SOFTWARE

Primer3 Input	www.simgene.com
SDS v2.4 (7900HT Fast Real-Time PCR System)	Appplied Biosystems
BLAST	http://genome.brc.mcw.eduEnsembl
Genome Browser	http://ensembl.org
UCSC Genome Browser	www.genome.ucsc.edu
PubMed	www.ncbi.nlm.nih.gov/entrez
Atlas of Oncology	www.atlasgeneticsoncology.org The
American Cancer Society	www.cancer.org
The Leukemia National Cancer Institute	www.cancer.gov
The Leukemia & Lymphoma Society	www.lls.org

3.2. GENERAL CELL CULTURE METHODS

For washing and harvesting, human cells were centrifuged using the general cell program: 3 min, 200xg, 37ºC. Cells were cultured at 37ºC, 5% CO_2 and 95% relative humidity in an incubator.

3.2.1. Culture and passaging of 293T cells

293T cells were cultured in DMEM (Gibco, USA) (Table 3-1) routinely supplemented with 10% FBS (Gibco, USA) and 1% antibiotics (penicillin and streptomycin) in a P150 cell culture plates.

Table 3-1│ Culturing and passaging of 293T cells.

Cell line	Medium	Passaging
293T	DMEM	Splitting by trypsination

Cell cultures were split 1:3 every 3-4 days (until reached 60-70% confluence). Cells were washed once with PBS and disaggregated by incubation with 0.05% Trypsin/0.02% EDTA/PBS (2 mL per 75 cm^2 vessel area) at 37ºC for 5 min until cells detached. Trypsin was then inactivated by adding 6 mL medium with 10% FBS.

3.2.2. Culture Conditions and Passaging of hMSC cells

Human MSCs were cultured in DMEM-F12 (Gibco, USA) (Table 3-2) routinely supplemented with 10% FBS (Gibco, USA), $_L$-glutamine (2mM) and 1% antibiotics (penicillin and streptomycin) in a T175 cm^2 culture flasks. Media supplements were purchased from Gibco. FBS was heat inactivated for 30 min at 56ºC before use.

Table 3-2│ Culturing and passaging conditions of hMSCs.

Cell line	Medium	Passaging
hMSCs	DMEM-F12	Splitting by trypsination

Cell cultures were split 1:4 every 4 days. Cells were washed once with PBS and disaggregated by incubation with 0.05% Trypsin/0.02% EDTA/PBS (3 mL per 75 cm^2 vessel area) at 37ºC for 5 min until cells detached. Trypsin was then inactivated by adding 6 mL medium with 10% FBS.

3.2.3. Inactivation and Preparation of feeders

Human MSCs were detached and resuspended in 40 mL medium with 10% FBS at the density approx. $15x10^4$ / mL and then irradiated with a dose 40 Gy of ionizing radiation. Cells were centrifuged, resuspended in a fresh medium and plated into T25 cm^2 cell culture flasks at the density $15x10^4$ / flask. Inactivated feeders were plated the day before splitting of hESCs and kept in an incubator at conditions mentioned above.

3.2.4. Preparation of human MSC conditioned medium (MSC-CM)

Human MSCs were split (at the density $2x10^6$/ T175 cm^2 flask) and cultured in a standard medium overnight to let the cells attach. After 24h, hMSC medium was removed and replaced with complete hESC medium supplemented with 4 ng/mL bFGF. After 24h the medium was collected and fresh complete hESC medium was added again. The cells were cultured during 6 days and the MSC-CM was collected daily and stored at 4ºC. Subsequently, MSC-CM medium was filtered through sterile 0.22 μm filter and stored at -80ºC.

3.2.5. Culture of hESCs on feeders

If not otherwise indicated, hESCs were cultured in KO-DMEM (Gibco, USA) (Table 3-3) routinely supplemented with 20% KO-SR (Gibco, USA), L-glutamine (2mM), MEM Non-essential Aminoacids (Gibco,USA), 50 μM β-mercaptoethanol and 8 ng/mL basic fibroblast growth factor (bFGF, IBIAN, Spain). The medium was changed daily.
Cells were split 1:3 every 6-7 days. Cell cultures were washed with KO-DMEM medium (without complements) and incubated with Collagenase IV (200 U/mL, 2 mL / flask) at 37ºC for 5 min. Subsequently, cells were washed once again and a fresh complete KO-DMEM medium was added. Human ESC colonies were cut and detached by scrapping under a phase contrast microscopy and then split into new T25 cm^2 flasks with fresh feeders.

Table 3-3| Culturing and passaging conditions of hESCs.

Medium		Flask	Passaging
KO-DMEM		T25 cm^2	Splitting by scrapping
KO-SR	20%		
$_L$-glutamine	5%		
NA-AA	5%		
β-mercaptoethanol	50 μM		
bFGF	8 ng/mL		

3.2.6. Feeder-free culture of hESCs

For genetic manipulation and hematopoietic differentiation, hESCs were maintained undifferentiated in a feeder-free culture. Briefly, hESCs were cultured in Matrigel (BD Biosciences, USA) – coated T25 cm^2 flasks in human MSC conditioned medium (MSC-CM) supplemented with 8 ng/mL bFGF. Media was changed daily and the cells were split weekly by dissociation with 200 U/mL of collagenase IV. Human ES colonies were visualized daily by phase contrast microscopy.

3.2.7. Freezing and thawing cells

Human MSCs were harvested and suspended at $2x10^6$ /mL in 900 μL ice cold FBS including 10% DMSO. After inverting the mix and transferring into cryovials, 100 μl DMSO (10% final) was added.

Human ESCs were harvested and suspended at 1 T25cm^2 flask / vial in 500 μl freshly prepared freezing medium 1 (FM 1) (Table 4-3). After inverting the mix and transferring into cryo-vials, 500 μl freezing medium 2 (FM2) (Table 3-4) was added.

Table 3-4| Freezing media for hESCs.

FM1	FM2
500 μL FBS + 500 μL KO-SR	800 μL FM1 + 200 μL DMSO

Tubes were rapidly inverted to mix cells properly. To allow gradual freezing at a rate 1ºC/min, the cryo-vials were placed into isopropanol-filled cryo-containers (Nalgene) and frozen at -80ºC for 24h. For long-term storage, the tubes were transferred in liquid nitrogen (-180ºC).

3.2.8. Control of genomic stability

For karyotype analysis cells were cultured in Matrigel-coated T25 cm^2 flasks for 5-6 days (60-70% of confluence). The day of harvest Colcemid was added (0.1 μg/mL, KaryoMAX, Invitrogen, USA) and the cells were incubated for 4h. Subsequently, the cells were detached using TrypleSelect (Gibco, Invitrogen, USA), transferred to 15 mL Falcon tube and gently triturated to obtain single cell suspension. An equal volume of hESC complete medium was added to inactivate the Trypsin. Then, the cells were centrifuged and 10 mL of prewarmed 0.075 M KCl was added. After 15 min incubation, 3 drops of fresh fixative (acetic acid: ethanol, 3:1) was added and the cells were centrifuged. Using plastic Pasteur pipette, 5-10 mL of fresh fixative wad added dropwise while vortexing the cells at lowest speed. Such fixed cells were stored at 4ºC until slide preparation and metaphase analysis.

3.2.9. Control of pluripotency

RT-PCR

Total RNA was isolated from undifferentiated hESCs and then cDNA synthesis was done (details given in section 3.6). RT-PCR was performed to detect the presence of four pluripotency genes: *NANOG, SOX2, OSCT3/4, REX1*. The primers used for RT-PCR are given in section 3.1.6.

Immunofluorescent staining of pluripotency markers

As an additional method to confirm undifferentiated state of hESCs, immunofluorescent staining was used. The cells were cultured on Matrigel-coated chamber slides for 3-4 days. The day of harvest the cells were washed with PBS and fixed with 4% PFA for 15 min at 37ºC. After washing 3 times, PBS-0.1% Triton was added and the cells were incubated for 15 min at RT to permeabilize the cell membrane. Cells were washed and the nonspecific binding sites were blocked by 1h incubation with PBS-5% BSA at RT. The staining with primary antibodies (OCT3/4, TRA-1-81) was performed overnight at 4ºC in darkness. The day after, the cells were washed 3 times with PBS and the secondary antibodies were added and the cells were incubated for 2h at RT. Subsequently, the cells were washed under low light and DAPI (Invitrogen, USA) nuclear staining was performed for 10 min at RT. Cells were analyzed using confocal microscope.

3.3. ESTABLISHMENT OF TRANSGENIC CELL LINES

3.3.1. Lentiviral transduction

For lentiviral transduction cells were cultured on Matrigel-coated P6-well plates for 2-3 days (until 60-70% confluence) and then lentiviral transduction was performed (Figure 3-5). Briefly, the cells were washed with KO-DMEM medium without complements and then exposed to 8 μg/mL to Polybrene during 15 min at 37ºC to make the cell membrane more permeable. After 15 min of incubation, a 500 μL of concentrated LV was added (resuspended in KO-DMEM medium complemented with 8 ng/mL bFGF) and the cells were incubated overnight at standard culture conditions. The day after, the cells were washed and fresh complete medium was added. 48 hours after medium change the expression of eGFP in hES cells was controlled under fluorescent microscope and/or the process of selection was started.

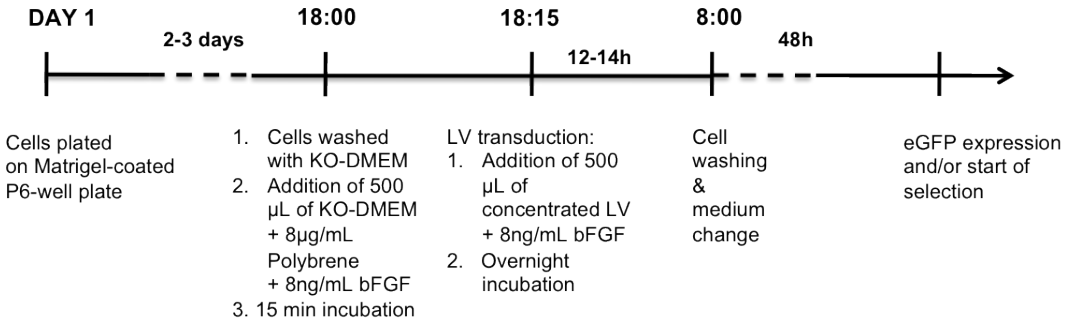

Figure 3-5│ Schematic representation of the lentiviral transduction of hESCs.

3.3.2. Selection of positive clones

First two lentiviral vectors (NEO and MLL-AF9) used for transduction of hESCs contain the Neomycin resistance cassette. Therefore, the selection of positive clones was performed by treatment of transduced cells with G418 (Sigma-Aldrich, Germany) at concentration 100 mg/mL during 5 weeks.

Since FLT3 vectors (FLT3-WT and FLT3-ITD) contain EGFP cassette as a marker, the selection was performed manually under fluorescent microscope basing on EGFP expression.

3.4. HEMATOPOIESIS-DIRECTED DIFFERENTIATION FROM hESCs

3.4.1. Embryonic Body formation and hematopoietic differentiation protocol

For human embryonic body (hEB) formation human ES cells were cultured into Matrigel-coated T25 cm^2 flasks in MSC-CM. Confluent undifferentiated hES cells were treated with collagenase IV (Gibco, USA) and scrapped off from the flasks and then transferred to low-attachment P6-well plates (Corning, USA) To allow hEB formation, cells were incubated overnight in aggregation medium (AgM) (Table 3-5). The next day, half of medium was changed and replaced by fresh induction medium (IM) (Table 3-6).

Table 3-5│ Composition of the aggregation medium (AgM).

Reagent / Volume of medium	4 mL
Stem-Pro34-SFM medium (+ delivered nutrition supplement, Invitrogen)	3.3 mL
FBS Hyclone (final concentration 10%, Cultek??)	0.4 mL
Vitamin C (final concentration 50 µg/ml, Sigma)	4 µL
MTG (stock solution 100 mM, 1-Thioglycerol, Sigma)	4 µL
L-Glutamine (final concentration 1%)	40 µL
Pen/Strep (final concentration 1%)	40 µL
BMP-4 (stock solution 5 ng/µL, final concentration 50 ng/ µL)	40 µL
VEGF (stock solution 10 ng/µL, final concentration 50 ng/ µL)	20 µL

Table 3-6│ Composition of the induction medium (IM).

Reagent / Volume of medium	4 mL
Stem-Pro34-SFM medium (+ delivered nutrition supplement, Invitrogen)	3.3 mL
FBS Hyclone (final concentration 10%)	0.4 mL
Vitamin C (final concentration 50 µg/ml, Sigma)	4 µL
MTG (stock solution 100 mM, 1-Thioglycerol, Sigma)	4 µL
L-Glutamine (final concentration 1%)	40 µL
Pen/Strep (final concentration 1%)	40 µL
BMP-4 (stock solution 5 ng/µL, final concentration 50 ng/ µL)	40 µL
VEGF (stock solution 10 ng/µL, final concentration 50 ng/ µL)	20 µL
SCF (stock solution 10 ng/µL, final concentration 40 ng/ µL)	16 µL
TPO (stock solution 10 ng/µL, final concentration 40 ng/ µL)	16 µL
FLT3 (stock solution 10 ng/µL, final concentration 40 ng/ µL)	16 µL

Human Embryonic Bodies were cultivated 15 days to perform analysis (Figure 3-6).

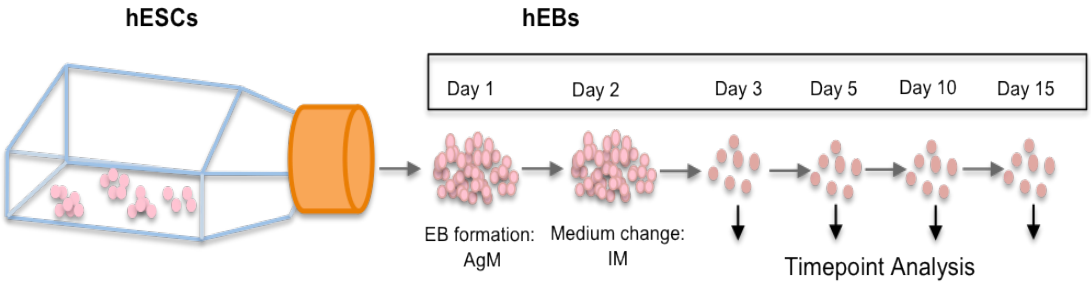

Figure 3-6| Schematic representation of the hematopoietic differentiation protocol of hES cells by Embryonic Body formation.

3.4.2. Disaggregation of Embryonic Bodies

Human EB were dissociated using StemPro Accutase (StemCell Technologies, Canada) for 1h at 37ºC and gentle pipetting at days 3, 7, 10 and 15 of development. Cells were washed with PBS and passaged through a 70-μm cell strainer to achieve single cell suspension.

3.4.3. Colony Forming Assay

Human clonogenic progenitor assay was performed by plating 1000 cells from day 15 hEBs into Methylcellulose H4230 (Stem Cell Technologies, Vancouver, Canada) supplemented with recombinant human growth factors: 50ng/ml SCF, 3 units/mL erythropoietin, 10ng/mL GM-CSF, and 10ng/mL IL-3. Cells were incubated at 37^{o}C in a 5% CO_2 humidified atmosphere and colonies counted at day 14 of CFU assay using standard morphological criteria.

3.5. GENERAL MOLECULAR BIOLOGY

3.5.1. Bacterial culture

E.coli strains were streaked out on solid LB-agar with appropriate antibiotics and grown overnight (O/N) at 37°C. Single colonies were then picked into liquid LB-medium containing the ampicillin and grown overnight at 37°C with shaking at 200 rpm.

LB-medium:	10 g	NaCl
	10 g	Bacto Tryptone (Difco)
	5 g	Yeast extract
	Add ddH_2O to 1000 ml, autoclave	

LB-agar plates:	15 g	Agar
	10 g	NaCl
	10 g	Bacto Tryptone (Difco)
	5 g	Yeast extract
	Add ddH_2O to 1000 ml, autoclave, cool to 50°C and add the appropriate antibiotic	
	Pour the agar solution into 10 cm Petri dishes, and store inverted at 4°C	

3.5.2. Transformation of chemically competent *E.Coli*

Chemically competent *E.coli* (50 µL) were thawed on ice, 1-25 ng plasmid DNA in 2-5 µL volume was added and the suspension was mixed gently and incubated on ice for 20 min. Cells were heat-shocked in a water bath at 42°C for 30 s, immediately cooled on ice for 2 min and 250 µL SOC medium was added. To express the resistance, bacteria were incubated for 1 h at 37°C with shaking and 50-150 µL of the transformation were plated and incubated overnight at 37°C on LB-agar containing the antibiotic necessary for selection of transformed cells.

SOC medium	20 g	(2%)	BactoTrypton (Difco)
	5 g	(0.5%)	BactoYeastExtract (Difco)
	0.6 g	(10 mM)	NaCl
	0.2 g	(3 mM)	KCl
	Add ddH_2O to 1000 ml, autoclave and add to the cooled solution:		
	10 ml	(10 mM)	MgCl2 (1 M), sterile filtered
	10 ml	(10 mM)	MgSO4 (1 M),sterile filtered
	10 ml	(20 mM)	Glucose (2 M), sterile filtered

3.5.3. Glycerol stock

For long-term storage, bacteria were stored at -80°C in 20% glycerol by adding 600 µL liquid culture to 200 µL of 80% glycerol.

3.5.4. Plasmid isolation from *E.coli*

To check if the isolated single *E.coli* colonies contained the correct plasmid, DNA mini-prep was carried out using Qiagen Plasmid Mini Kit following the supplied instructions. To isolate larger amounts of ultra pure DNA (100µg) for transfection experiments, plasmids were isolated using the endotoxin-free Qiagen Plasmid Maxi Kit (Qiagen, Germany).

3.5.5. Construction of lentiviral vectors

All lentiviral vectors were kindly provided by Dr. Pablo Menéndez (Genyo, Granada, Spain). Briefly, the *MLL-AF9* cDNA (*MLL* exon 9 was fused to *AF9* exon 9) was subcloned into the PmeI site of pRRL-EF1α-PGK-NEO vector. In the case of *FLT3* lentiviral vectors, the *FLT3-WT* and *FLT3-ITD* cDNAs were subcloned into PmeI site of pRRL-EF1α-PGK-EGFP vector. Resuming, the following vectors were used in this study (see also Chapter 3.1.8.):

pRRL-EF1α-PGK-NEO (control, NEO)
pRRL-EF1α-MLL-AF9-PGK-NEO (MLL-AF9)
pRRL-EF1α-FLT3-WT-PGK-EGFP (FLT3-WT)
pRRL-EF1α-FLT3-ITD-PGK-EGFP (FLT3-ITD)

3.5.6. Preparation of lentivirus

Viral particles pseudotyped with VSV-G were generated on 293T cells by standard calcium-phosphate transfection protocol.

Following solutions were used:

2xHBS (HEPES-buffered saline)

NaCl (MW 58.44 g/mol)	8.1 g
KCl (74.56 g/mol)	0.38 g
$Na_2PO_4 \cdot H_2O$ (MW 177.99 g/mol)	0.1 g
HEPES (MW 238.30 g/mol)	5.1 g
D-(+)-Glucose (Sigma)	1.0 g
H_2O (MilliQ)	500 mL

Dissolve all reagents in 500 ml H_2O and adjust pH at 7.05 with 0.5 N NaOH. The pH 7.05 is very important; below 7 precipitates ill not form and above 7.08 precipitates will be coarse and transfection will fail. Filter the solution with 0.22 µm in the hood (after filtration you still may want to confirm pH). Make suitable aliquots of 2xHBS and store at -80°C. Once thawed, 2xHBS solution can be stored for several weeks (up to 3 months) at 4°C without observing any change in the transfection efficiency.

2M $CaCl_2$

$CaCl_2$ (MW 147)	14.7 g
H_2O (MilliQ)	50 mL

Dissolve $CaCl_2$ in 50 ml H_2O. Then, filter with 0.22 µm in the hood. Store adequate aliquots at -80°C. Once thawed $CaCl_2$ solution can be stored up to 3 months at 4°C.

3.5.7. Production of lentiviral particles

The packaging cells (293T) were plated out the day before transfection at confluence ~ $5x10^6$/p150 plate and used at 60-70% confluence. Just prior transfection, the medium was carefully replaced with fresh DMEM-10% FBS containing 25 µM chloroquine (2.5 µL of 100 mM stock solution/ 10 mL) and the cells were incubated until the whole set-up was finished.

During incubation, the transfection mixture was prepared:

- 22.5 µg of LV of interest
- 7 µg of envelope plasmid (pMD2G)
- 14 µg of packaging plasmid (pPAX2)
- 389 µl of 2M $CaCl_2$

  with the final volume 3 ml.

An equal volume of 2x HBS was added dropwise to the transfection mixture while vortexing. Subsequently, 6 mL of transfection mixture was added to the cells in a dropwise manner (Figure 3-7). After ~ 15 min, a black crystalline precipitates were observed under microscope. The cells were incubated ~8-12h at 37ºC and then the medium was replaced with fresh DMEM-10% FBS to remove chloroquine.
The lentiviral supernatant from packaging 293T cells was collected after 24 and 48h with a sterile 10 ml syringe, filtered through 0.45 µm filter and stored at 4ºC prior to concentration.

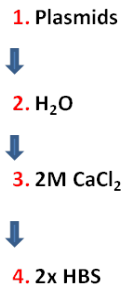

Figure 3-7 | Preparation of the transfection mixture.

3.5.8. Concentration and storage of lentivirus

Produced lentiviral supernatant was then concentrated by ultracentrifugation at 26.000 rpm for 2 h at 16ºC. After discarding of the supernatant, lentiviral pellet was then resuspended in 1 mL of cold and sterile KO-DMEM medium using a P1000 pipette and stored in cryotubes at -80ºC. Lentiviral supernatant was concentrated 16x.

3.5.9. Preparation and analysis of RNA

Total RNA was isolated using the Qiagen RNeasy Kit. RNA concentration was then determined with the NanoDrop spectrophotometer and quality was assessed by agarose gel electrophoresis or using the Agilent Bioanalyzer according to the manufacturer's instructions. Isolated RNA was stored at -80ºC for future analysis.

3.5.10. Preparation of cDNA

The cDNA was prepared from the total RNA isolated from all hES cell lines using High Capacity cDNA Reverse transcription Kit (Applied Biosystems, USA). The following protocol was used:

1. RNA samples were thawed on ice and then a solution of 1μg RNA in 10 μL nucelase-free H_2O was prepared and kept on ice.

2. A cold cDNA RT master mix was prepared:

10x RT buffer	2 μL
25x dNTP	0.8 μL
10x random primers	2 μL
Reverse transcriptase	1 μL
RNAse inhibitor	1 μL
Nuclease-free H_2O	3.2 μL
TOTAL	10 μL

3. After preparing of RNA solution and the cDNA master mix, the following cDNA reaction mix was prepared (by mixing and gentle pipetting):

10 μL of 2x RT master mix

10 μL of RNA solution (1μg)

4. The following reaction was set up and run at the thermocycler (Table 3-7):

Table 3-7 | PCR conditions for preparation of cDNA.

cDNA reaction				
Step 1	Step 2	Step 3	Step 4	Total
25°C	37°C	85°C	4°C	2h 12´
10 min	60 min	5 sec	∞	

5. Constructed cDNA (at concentration 100 ng) was stored at -20ºC for future analysis.

3.5.11. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) allows *in vitro* synthesis of large amounts of DNA by primed, sequence-specific polymerization of nucleotide triphosphates, catalysed by DNA polymerase (Mullis, Faloona et al. 1986). PCRs were generally performed in PCR tubes with a reaction volume of 20-100 µL. The nucleotide sequences of the utilized primers are given in section 3.1.6. The primer annealing temperatures varied between 57 and 65°C. General parameter settings for analytical PCR are summarized in Table 3-8.

Table 3-8 | Reaction parameter for analytical PCR.

PCR step		Cycling parameter
Initial melting		94ºC 5 min check!!
30-35 cycles	Melting	94ºC - 30 sec
	Annealing	60ºC - 50 sec
	Extension	72º - 50 sec
Final extension		72ºC - 10 min
Cool to		4ºC

Real time PCR

Quantitative Real Time PCR (qRT-PCR) was used for quantification of cDNA after reverse transcription (3.5.10). PCR reactions were performed using the QuantiFast SYBR Green Kit and TaqMan PCR Mix from Applied Biosystems (USA) in 96-well format adopted to the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The relative amount of amplified DNA is measured through the emission of light by the SYBR green dye, when it is intercalated in double stranded DNA. General parameter settings for qPCR are summarized in Table 3-9.

Reaction setup: 5 µl SYBR Green mix (2×) (QuantiFast, Qiagen)
2 µl ddH_2O
0.5 µl primer forward (10 µM)
0.5 µl primer reverse (10 µM)
2 µl DNA

Table 3-9| Reaction parameter for real time PCR.

PCR step		Cycling parameter
Initial melting		95ºC - 10 min
45 cycles	Melting	95ºC - 15 min
	Combined annealing and extension	60ºC - 1 min
Final cycle	Melting	95ºC - 15 min
	Combined annealing and extension	60ºC - 1 min
Melting curve		10-20 - min
		72ºC - 30 sec

To calculate amplification efficiency, a dilution series (1:10; 1:50; 1:100, 1:1000) of a suitable sample was additionally measured for each primer pair. The SDS v2.4 (Applied Biosystems, USA) software calculated automatically DNA amounts based on the generated *slope* and *intercept*. Specific amplification was controlled by melting-curve analysis and data were imported and processed in Microsoft Excel 2003. All samples were measured in triplicates and normalized to the GAPDH or the TBP housekeeper when analyzing mRNA expression.

3.6. CELL CYCLE ASSAY (IP)

Cell cycle analysis by quantitation of DNA was one of the earliest applications of flow cytometry. The DNA of mammalian, yeast and bacterial cells can be stained by a variety of DNA binding dyes. The premise with all of these dyes is that they are stoichiometric i.e. they bind in proportion to the amount of DNA present in the cell. In this way cells that are in S phase will have more DNA than cells in G1, will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content and these cells in G2 will be approximately twice as bright as cells in G1.

For cell cycle analysis, undifferentiated hESCs cultured in Matrigel-coated T25 flasks were harvested, washed with wash buffer (PBS-1% FBS) and then fixed with 70% cold ethanol added dropwise to ensure proper fixation of all cells and minimize cell clumping. The cells were fixed overnight at 4ºC. After fixation, cells were washed twice in wash buffer and centrifuged. Subsequently, the supernatant was removed and the cells were suspended in 300 μL propidium iodide (PI 50 μg/mL) and stained for 48h at 4ºC. To ensure that only DNA is stained, cells were treated with Ribonuclease (100 μg/mL final concentration; Qiagen, Germany).

3.7. FLOW CYTOMETRY ANALYSIS

A single cell suspension achieved by hEB disaggregation was stained with following antibodies: anti-CD31, anti-CD34, anti-CD45 and DAPI. Live cells identified by DAPI exclusion were analyzed using FACS-Fortessa flow cytometer (BD Biosciences, USA). Hemogenic precursors with hemangioblastic precursors were identified as $CD34^{+}CD31^{+}CD45^{-}$. Immature and mature blood cells were identified as $CD45^{+}CD34^{+}$ and $CD45^{+}CD34^{-}$, respectively, (Figure 3-8).

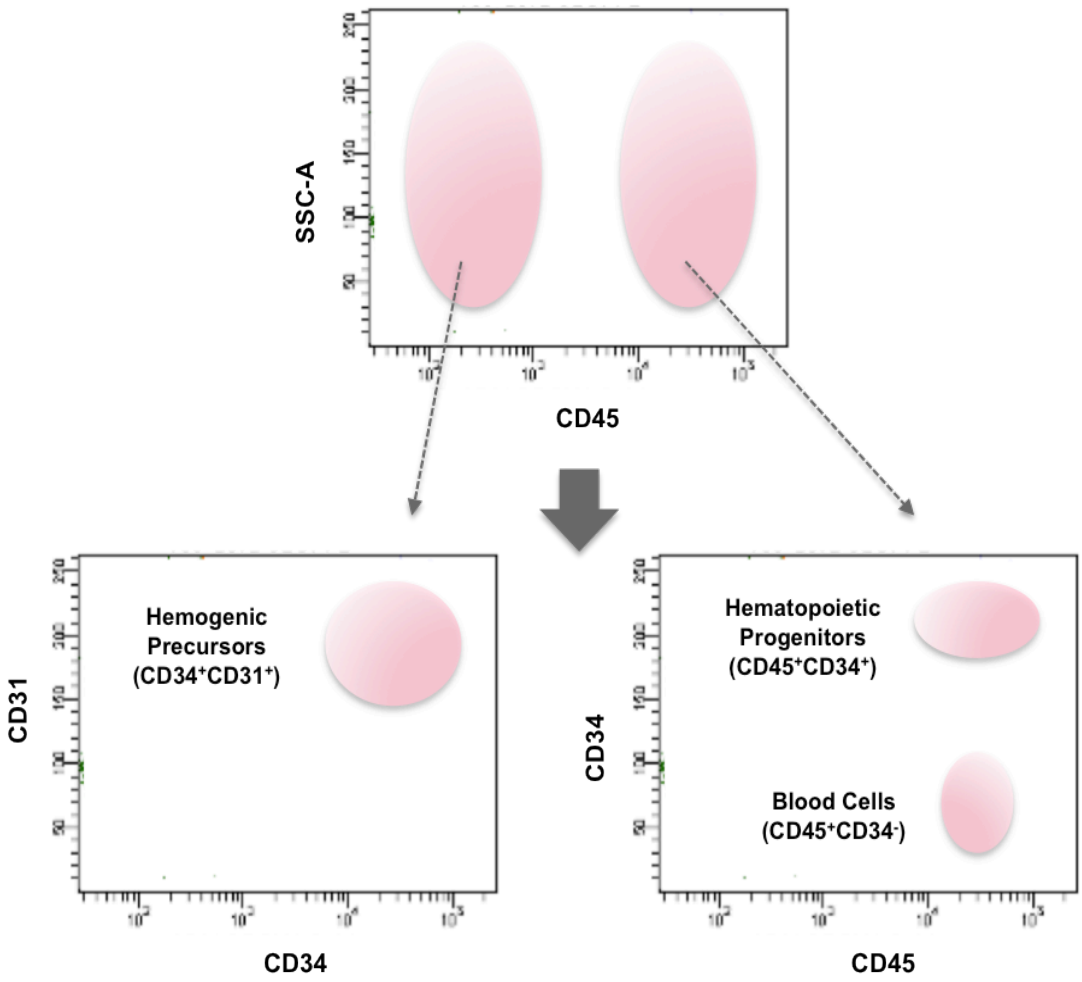

Figure 3-8 | Schematic representation of the FACS gating.

RESULTS

4.1. CREATING A HUMAN ESC MODEL EXPRESSING THE *MLL-AF9* FUSION GENE AND THE *FLT3-ITD* GENE AS A SECONDARY LEUKEMOGENIC HIT

Leukemias harboring the *MLL-AF9* fusion gene are associated with a dismal prognosis and their clinical consequences are well characterized. Concerning the biological processes affected by this fusion gene a little is known and our understanding of its transformation capacities is limited. It is also not known at which stage of hematopoietic differentiation this translocation can occur and which is the critical one in the process of leukemiogenesis.

FLT3, a member of the receptor tyrosine kinase (RTK) class III, is preferentially expressed on the surface of a high proportion of acute myeloid leukemia (AML) and B-lineage acute lymphocytic leukemia (ALL). A mutated form of *FLT3* gene, *FLT3-ITD* has been found in approximately 30% of adult AML patients and in approximately 10% of childhood AML patients. An interaction of FLT3 and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal hematopoietic cells but also leukemia cells (Stubbs, Kim et al. 2008).

Human Embryonic Stem Cells (hESCs) are becoming a powerful tool for modeling human diseases in which pathogenesis and progression may not be fully recapitulated with the use of patient samples or mouse models. It is well known, that the process of leukemogenesis manifests as altered cell differentiation. Having this in mind, hematopoiesis-directed differentiation of hESCs could become a promising strategy to study the onset of hematopoiesis, especially the emergence of the earliest events leading to the specification of both normal and abnormal hematopoietic tissue (Lensch and Daley 2006).

To date, no study has explored the effects of *MLL-AF9* leukemic fusion gene during hESC-derived hematopoietic development. Here we explored *in vitro* the impact of the enforced expression of *MLL-AF9* and *FLT3* genes on the onset of hematopoietic differentiation from human Embryonic Stem Cells.

4.1.1. ESTABLISHMENT OF A STABLE CELL LINE EXPRESSING THE *MLL-AF9* FUSION GENE

Human embryonic stem (hES) cells are derived from the pluripotent cells of the inner cell mass of the blastocyst (Thomson, Itskovitz-Eldor et al. 1998; Reubinoff, Pera et al. 2000). These cells can potentially proliferate indefinitely in culture; yet still retain a normal karyotype and the potential to differentiate into any cell type. Hence hES cells are expected to have far-reaching applications in regenerative medicine and basic

research. However, the exploitation of the remarkable potential of hES cells largely depends on the development of technologies that will allow efficient manipulation of the cells in vitro.

In this study, we have chosen to use lentiviral vectors that are derived from human immunodeficiency virus type 1 (HIV-1), given their potential to promote efficient transduction and stable transgene expression in a variety of cell types in vivo and in vitro (Naldini, Blomer et al. 1996; Blomer, Naldini et al. 1997; Kafri, Blomer et al. 1997). The lentiviruses offer advantages over other members of the retrovirus family. First, whereas most retroviruses infect only dividing cells, the lentiviruses can also infect nondividing cells. Second, although the use of retroviral vectors for gene delivery into murine ES cells proved ineffective because of silencing of transgene expression (Jahner, Stuhlmann et al. 1982; Niwa, Yokota et al. 1983), 'gene silencing' was not observed upon transduction of murine ES cells by an HIV-1 vector (Hamaguchi, Woods et al. 2000; Pfeifer, Ikawa et al. 2002). It has been reported, that modified self-inactivating (SIN) derived vectors HIV-1 are efficient tools for stable genetic modification of hES cells (Gropp, Itsykson et al. 2003). Transduction of hES cells by these vectors facilitates transgene expression that is maintained throughout prolonged cultivation as well as differentiation *in vitro* and *in vivo*.

In our study, human ESC line AND2 was transduced with either the empty lentiviral vector (NEO) or the *MLL-AF9*-expressing lentiviral vector (MLL-AF9) (Figure 4-1).

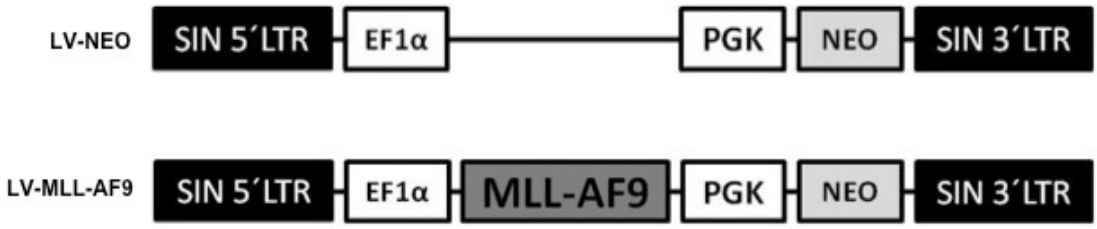

Figure 4-1 | Schematic representations of lentiviral vectors: LV-NEO and LV-MLL-AF9.

For lentiviral transduction cells were cultured on Matrigel-coated P6-well plates for 2-3 days (until 60-70% confluence) and then lentiviral transduction was performed (see Material and Methods, Figure 3-5).

First, the two lentiviral vectors (NEO and MLL-AF9) used for transduction of hESCs contain the Neomycin resistance cassette. Therefore, the selection of positive clones was performed by treatment of transduced cells with G418 at concentration 100

mg/mL during 5 weeks. After 5 weeks of G418 selection, typical neo-resistant hESC colonies emerged (Figure 4-2).

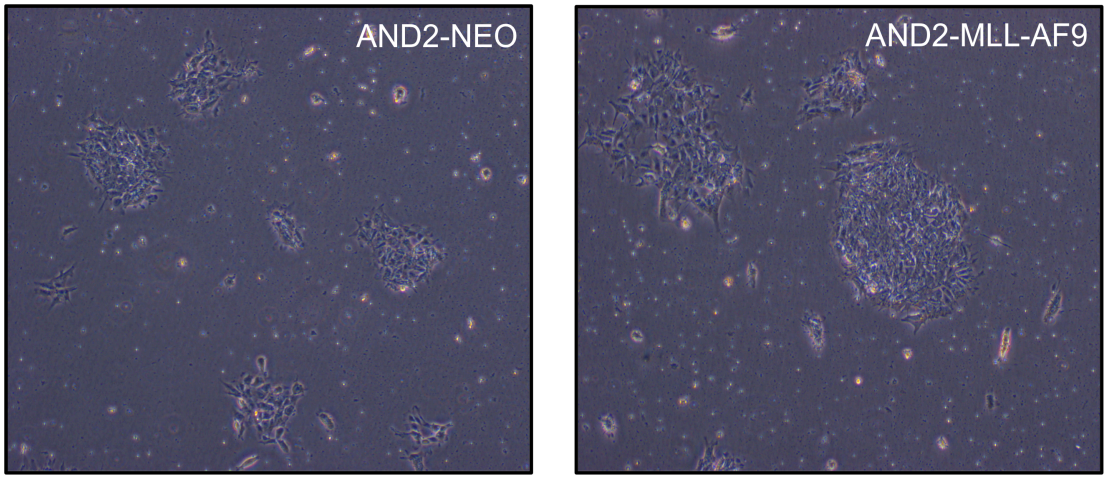

Figure 4-2| Morphology of NEO and MLL-AF9 hESC colonies after G418 selection.

Successful and stable integration and ectopic expression of the *MLL-AF9* transcript in transduced hESCs were confirmed firstly by RT-PCR (Figure 4-3) about 5 passages after selection. For the RT-PCR reaction, a plasmid DNA of the MLL-AF9 lentivirus was used as a positive control. As we can observe on Figure 4-3, the *MLL-AF9* transcript (334 bp) was detected in newly established hESC line (line 1).

Figure 4-3| RT-PCR confirming the integration of the *MLL-AF9* cDNA in transduced hESCs.

Secondly, the expression of the *MLL-AF9* fusion gene in transduced hESCs was confirmed by qRT-PCR (Figure 4-4). The leukemic cell line THP1 expressing the *MLL-AF9* fusion was used as a positive control. As we can observe, the transduction of hES cells was successful and we were able to express the *MLL-AF9* fusion.

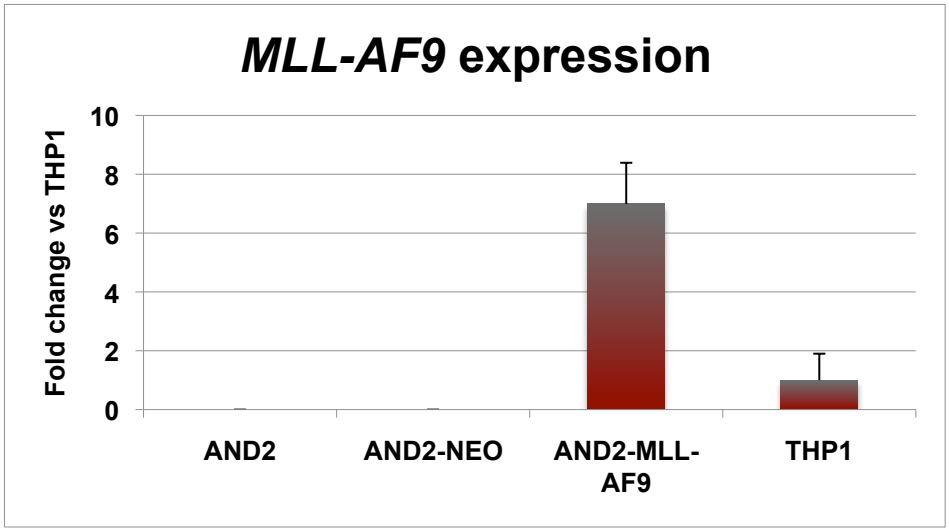

Figure 4-4 | qRT-PCR confirming the expression of the *MLL-AF9* transcript in transduced hESCs.

As a summary of this part, we were able to establish a hES cell line that ectopically and stably expressed the *MLL-AF9* fusion gene. This is the first known immortal established hESC cell line with this genotype. This cell line has become a unique cellular reagent since it is suitable for functional experiments, such as hematopoietic differentiation studies, among others.

4.1.2. ESTABLISHMENT OF STABLE CELL LINES EXPRESSING THE *FLT3-WT* AND *FLT3-ITD* GENES

The objective of our study was to investigate the effect of *MLL-AF9* fusion gene on the process of hematopoietic differentiation form hES cells. In addition, we decided to use *FLT3* gene and its mutated form *FLT3-ITD* as a second leukemic hit, basing on the fact that *FLT3-ITD* mutation has been found in around 30% of *MLL-AF9* leukemias.

After successful establishing of hESC line expressing the *MLL-AF9* vector (*MLL-AF9*, chapter 4.1.1), we transduced the cells with either the *FLT3-WT* lentivector (FLT3-WT) or the *FLT3-ITD* lentivector (FLT3-ITD) (Figure 4-5).

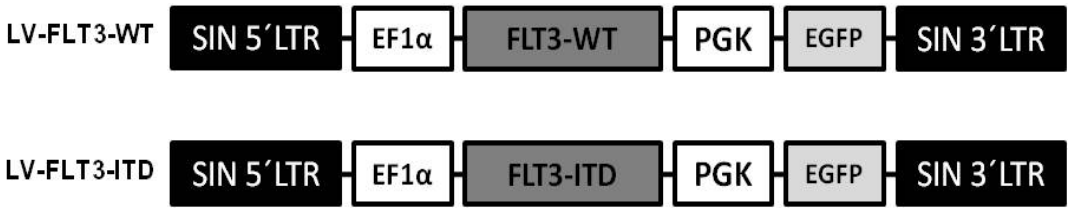

Figure 4-5 | Schematic representations of lentiviral vectors: LV-FLT3-WT and LV-FLT3-ITD.

Since both *FLT3* vectors (FLT3-WT and FLT3-ITD) contained EGFP cassette as a marker, the selection of positively transduced cells was performed manually under fluorescent microscope basing on EGFP expression. The expression of EGFP in hESCs was confirmed by fluorescent microscopy after 48 hours. As we can observe on Figure 4-6, the efficiency of lentiviral transduction was very high and reached 90-100%. Such transduced and selected cells were passaged into T25 culture flasks and subsequently cultured and expanded for further experiments (Figure 4-7).

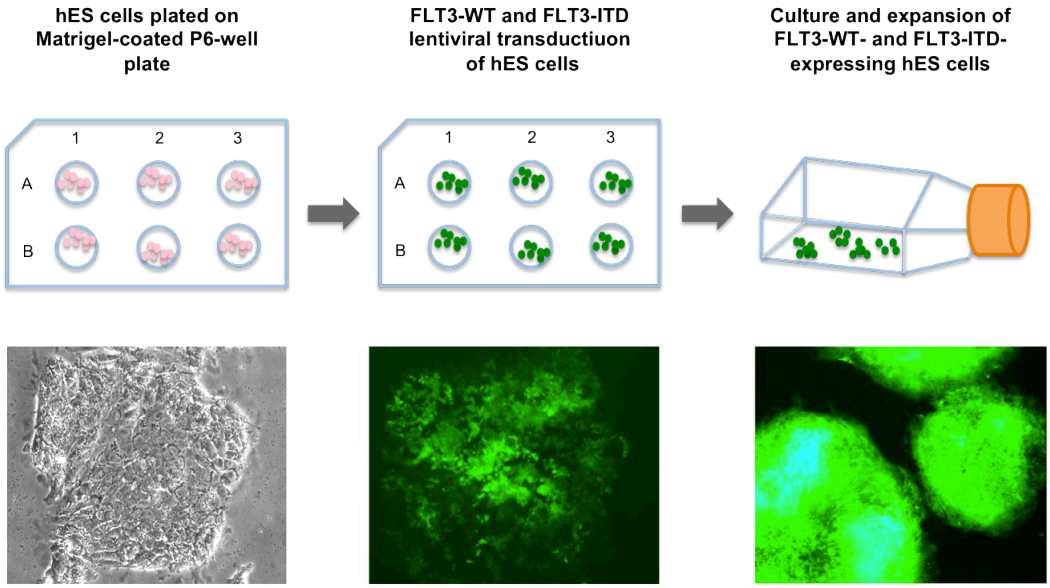

Figure 4-6 | Schematic representation of the selection of *FLT3-WT*- and *FLT3-ITD*-expressing hESC colonies.

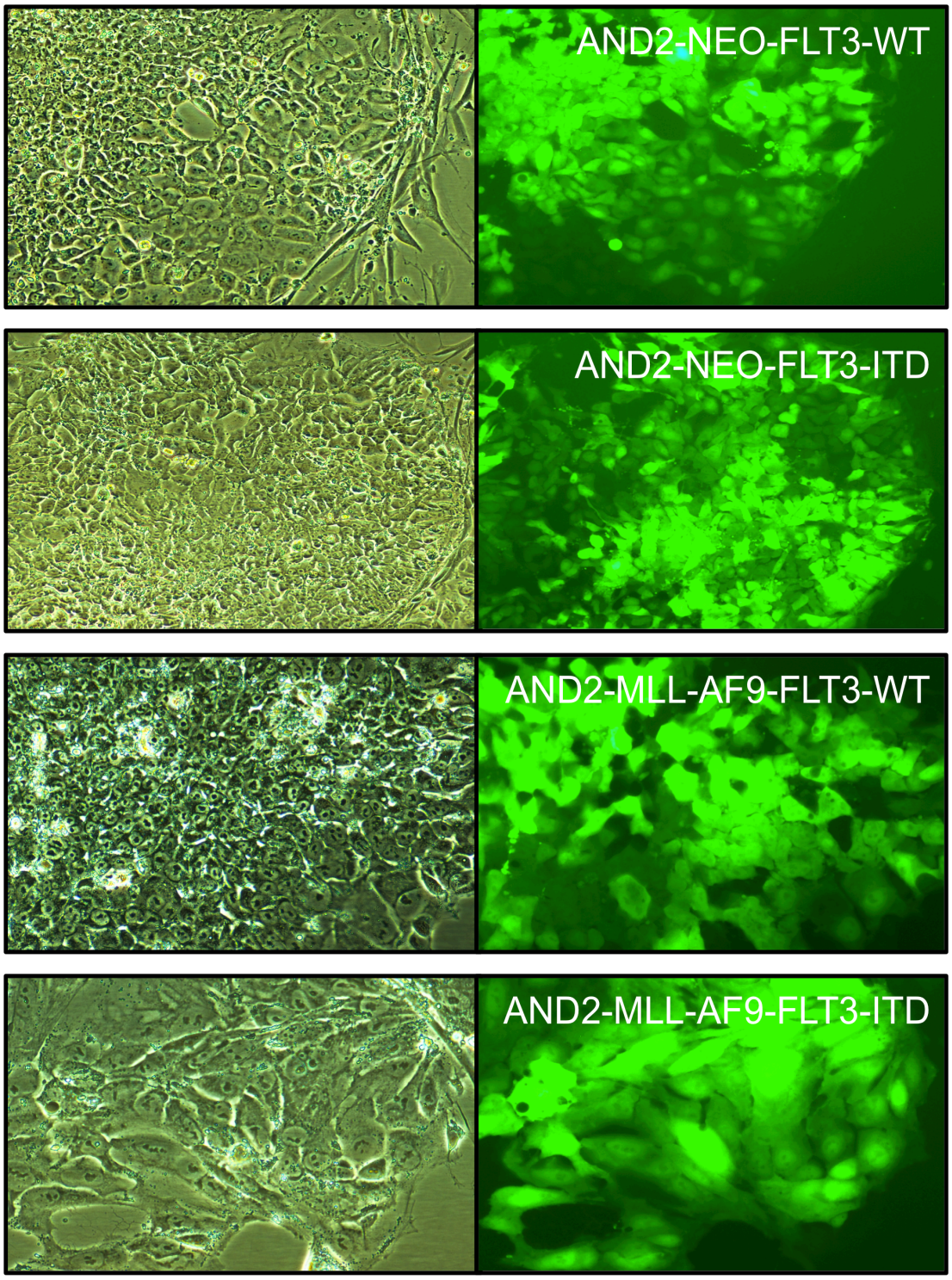

Figure 4-7 | Morphology of selected and expanded *FLT3-WT*- and *FLT3-ITD*-expressing hESC colonies.

Successful and stable integration of *FLT3* genes in the newly established hESCs was confirmed by RT-PCR in selected cultures 5 passages after the selection strategy. As we can observe on Figure 4-8, the endogenic *FLT3* (329 bp) was detected in all hESC lines (lines 1-7) and also in the case of positive control (line 8) (Control DNA IVSS0017, Invivoscribe, USA). As expected, the *FLT3-WT* (239 bp) and *FLT3-ITD* (259 bp) transcripts were also detected in the newly established hESC lines (lines 4 and 6, and lines 5 and 7, respectively). In the case of the negative control (Control DNA, IVS0000, Invivoscribe, USA), only the endogenic *FLT3* was detected.

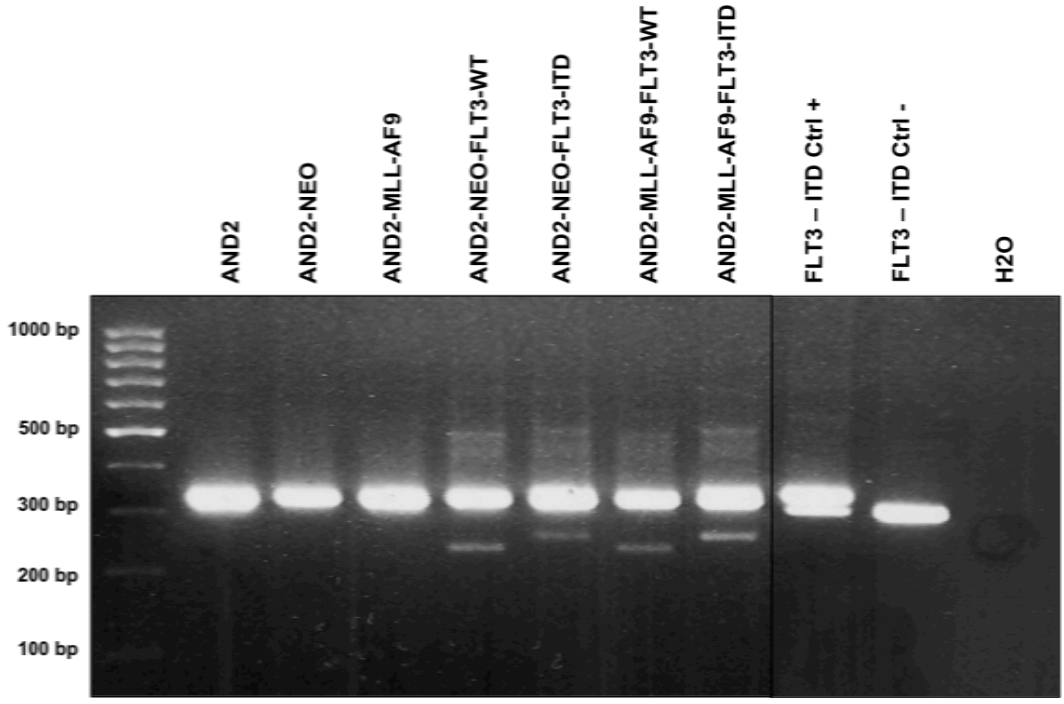

Figure 4-8 | RT-PCR confirming the integration of the *FLT3-WT* and *FLT3-ITD* transcripts in the transduced hESCs.

After the confirmation of the successful integration of the *FLT3-WT* and *FLT3-ITD* transcripts, we decided to measure the levels of their expression in the transduced hES cells. To measure the expression of the *FLT3-WT* transcript, we used standard *FLT3* PCR primers designed for qualitative RT-PCR that are available in the literature (van Dongen, Macintyre et al. 1999; van der Velden, Hochhaus et al. 2003). In the

case of the mutated *FLT3-ITD* form, we tried to use primers designed according to the *FLT3-ITD* sequence, but the quantitative PCR did not work. Based on the literature (Kottaridis, Gale et al. 2002; Stirewalt and Radich 2003; Yao, Nishiuchi et al. 2003) and the possible effect of *FLT3-ITD* mutation on the mRNA levels of endogenic *FLT3*, we assumed that the *FLT3-ITD* would deregulate its expression. Then, we decided to use this parameter (the endogenous gene) to confirm the successful expression of the *FT3-ITD* transcripts in the transduced hES cells.

As expected, in the case of *FLT3-WT*, we observed significantly higher levels of endogenic *FLT3* in the transduced hES cells (Figure 4-9, bars 3 and 6) when comparing with the wild type hESC line (bars 1 and 2, negative controls). In the case of *FLT3-ITD*, our conjecture was correct and we also observed the upregulation of the endogenic *FLT3* in the transduced hES cells (bars 4 and 7).

We also compared the levels of the expression of endogenic *FLT3* (our surrogate for *FLT3-ITD* expression) in our hESC model with some leukemic cell lines: THP1 is a cell line that harbors the *MLL-AF9* fusion; and MV4,11 and REH are cell lines that are commonly used as positive controls for *FLT3-ITD* and *FLT3-WT* transcripts, respectively. In line with previous observations, we demonstrated that the *MLL-AF9*-expresing hESC line also showed an upregulated level of endogenic *FLT3* (bar 5) and a level of expression that was similar to the leukemic THP1 cell line that bears the *MLL-AF9* fusion (bar 8).

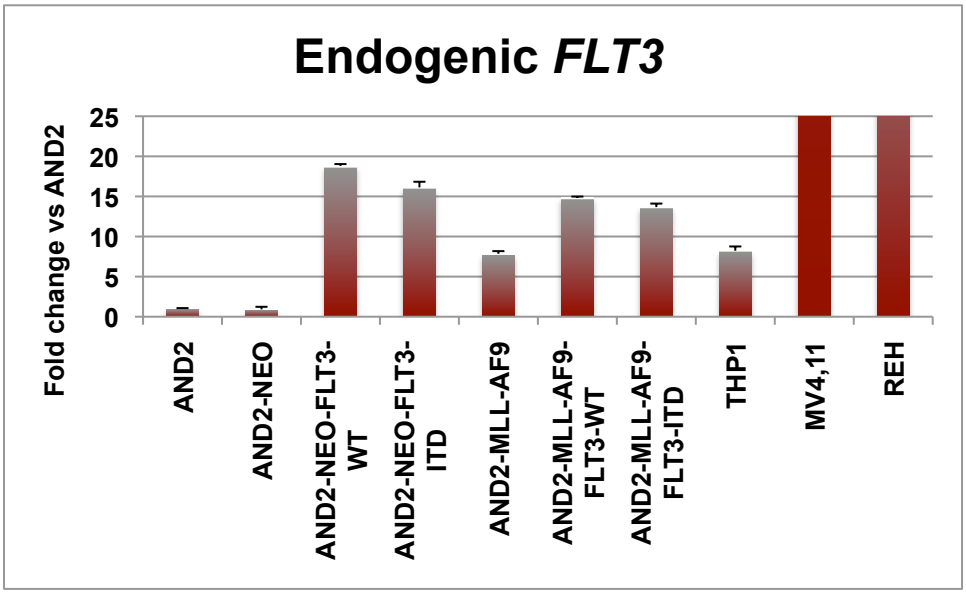

Figure 4-9| qRT-PCR confirming the upregulation of the endogenic *FLT3* upon ectopic expression of *FLT3* transcripts.

After establishment of all transgenic cell lines co-expressing both *MLL-AF9* and *FLT3-WT* and *FLT3-ITD* transcripts, the expression of *MLL-AF9* was monitored regularly by RT-PCR (Figure 4-10) and qRT-PCR (Figure 4-11).

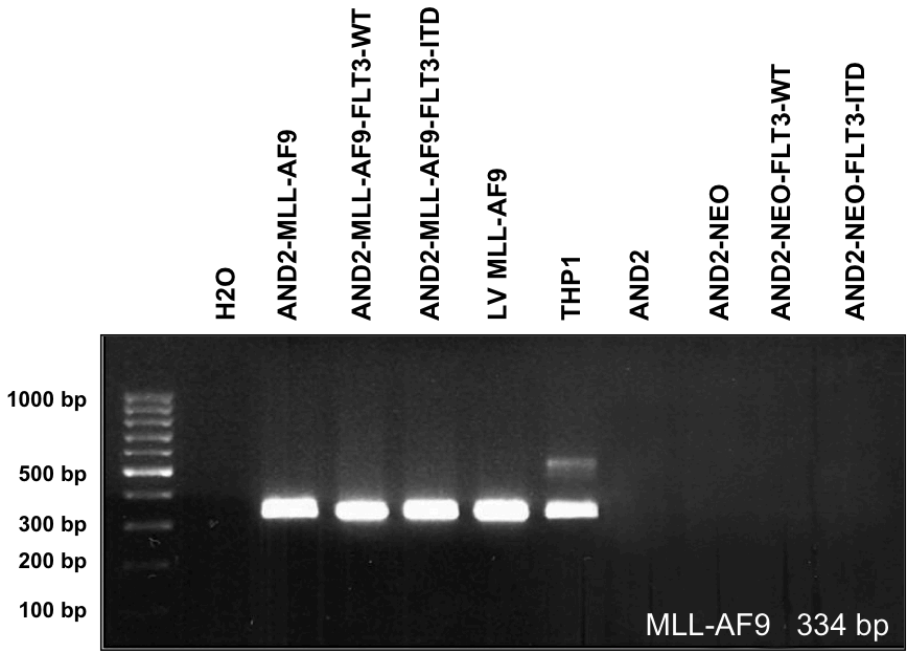

Figure 4-10 | RT-PCR confirming the integration of the *MLL-AF9* transcript in all transgenic hES cell lines.

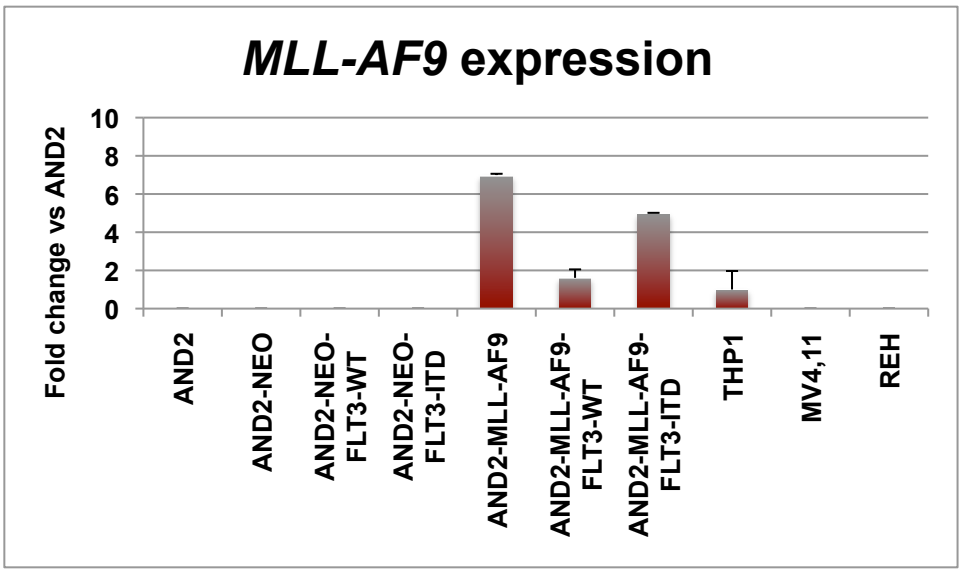

Figure 4-11 | qRT-PCR confirming the expression of the *MLL-AF9* transcript in newly established transgenic hESCs.

As a summary of this part, we were able to establish a hES cell lines that ectopically and stably co-expressed the *MLL-AF9* fusion gene and the *FLT3-WT*/FLT-*ITD* genes. These are the first known immortal established hESC cell lines with this genotype. These cell lines have become a unique cellular reagent since they are suitable for functional experiments, such as hematopoietic differentiation studies, among others. In the case of the co-expression of the *MLL-AF9* fusion gene with the *FLT3-WT* gene, we observed an unexpected down-regulation of the expression of the *MLL-AF9* transcript. Whether this down-regulation may be a direct effect of the *FLT3-WT* over-expression, remains an open question and requires further studies.

4.1.3. PLURIPOTENCY STATE OF NEWLY ESTABLISHED hES CELL LINES

One of the objectives of our study was to create a model of *MLL-AF9* leukemia based on human Embryonic Stem Cells. To do that, we had to introduce mentioned fusion gene into hES cells and create in that way a genetically modified hES cell lines. Since these new transgenic cell lines were supposed to be used for hematopoietic-directed differentiation, we had to be sure that they maintain their pluripotency, which would allow their differentiation into specific tissues. Therefore, we had to control regularly the pluripotency state of newly established hES cell lines. The most common way to do this control is checking for the expression of certain pluripotency markers specific for human ES cells, whose expression forms the basis for characterization and isolation of human ES cells. These markers may be monitored by their expression at the mRNA level as well as by their expression at the proteins level (they are proteins present in the cell membranes). Currently, there are few well-defined protein epitopes that definitely mark the pluripotency status of hES cells (Johnson, Shindo et al. 2008).

In our work, the newly generated hESC cell lines were first analyzed by RT-PCR for the expression of the most common pluripotency markers: Oct3/4, Rex1, Nanog and Sox2. As we can observe on the Figure 4-12, all transgenic hESC lines retained high expression of Oct3/4, Rex1, Nanog and Sox2.

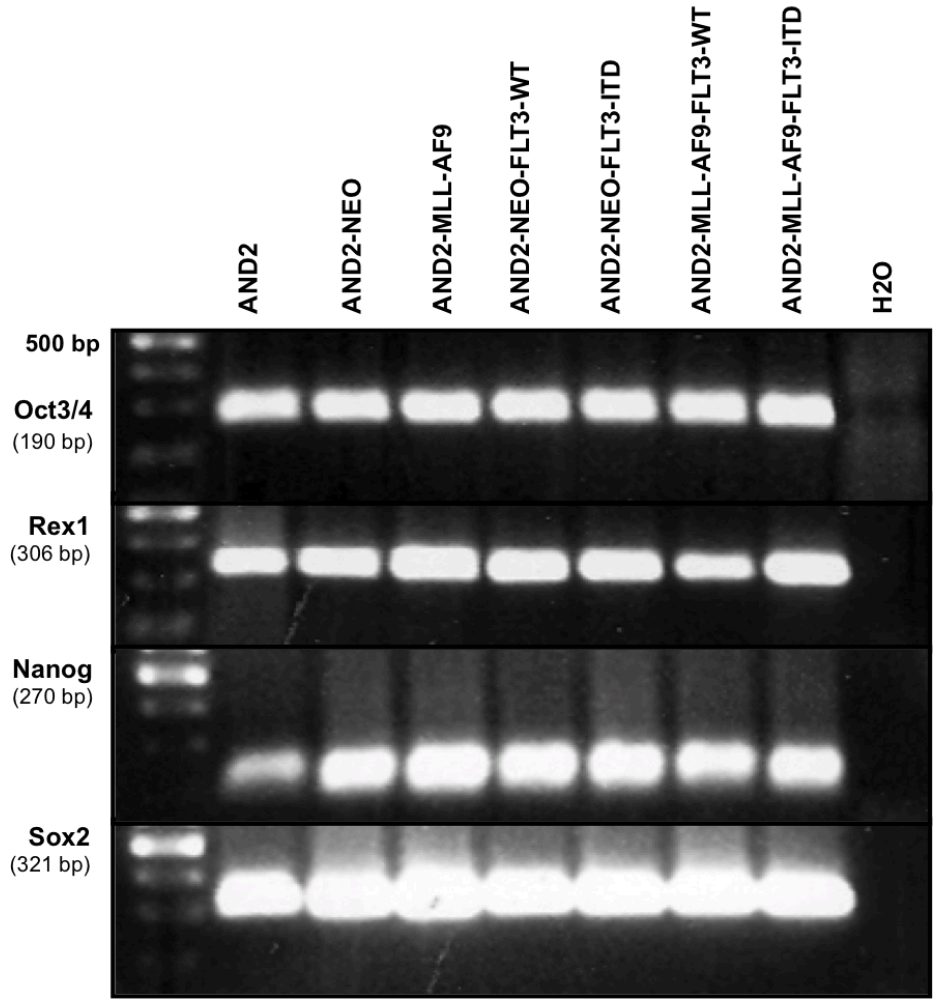

Figure 4-12 | RT-PCR showing the comparable mRNA expression levels of the pluripotency markers Oct3/4, Rex1, Nanog and Sox2 in the newly established transgenic hES cell lines.

The expression of pluripotency markers specific for human ES cells was also confirmed by immunofluorescent staining of OCT4 and TRA-1-81. As presented on Figure 4-13, the newly established hES cell lines also retained high expression of human ESC-associated antigens OCT4 and TRA-1-81.

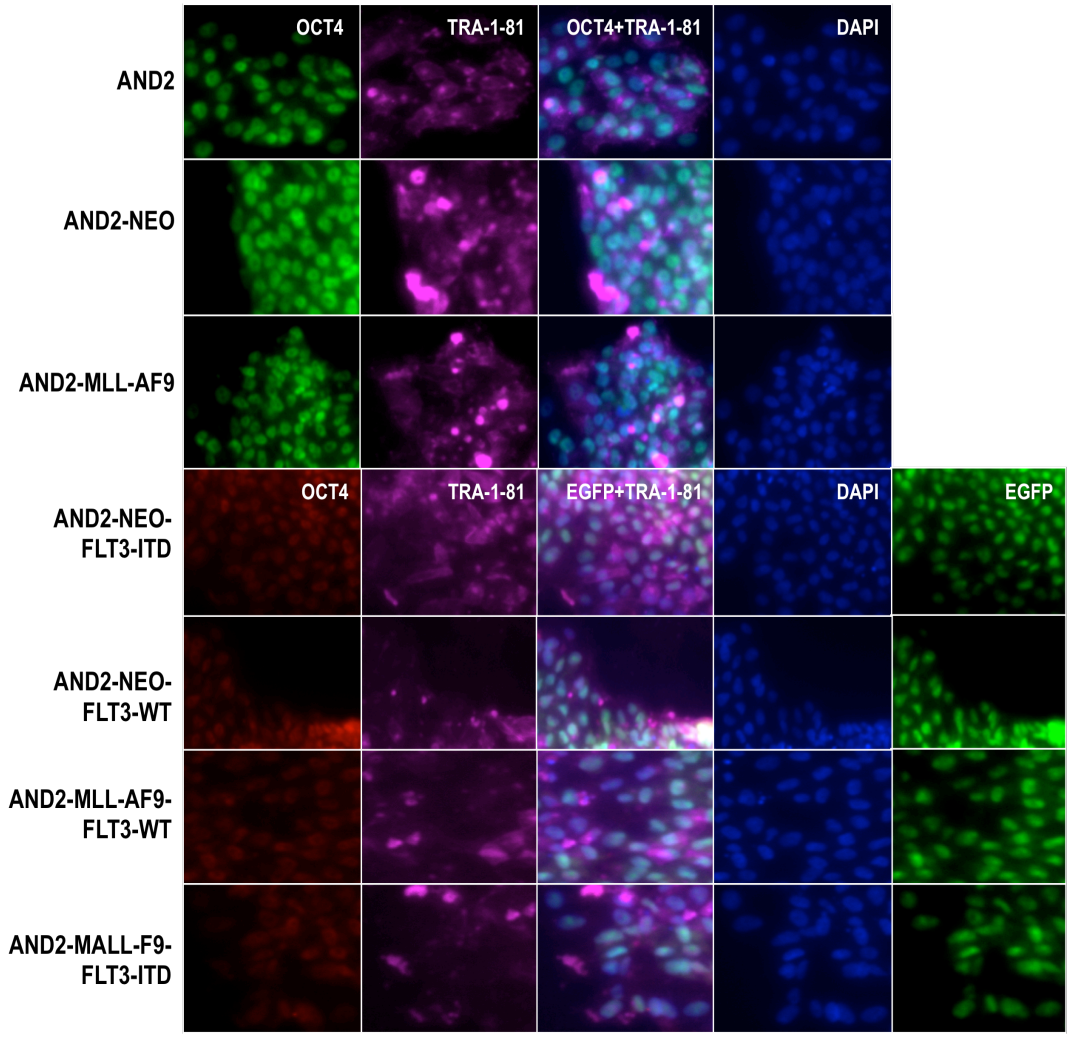

Figure 4-13 | Immunofluorescent staining of hESC-associated antigens OCT4 and TRA-1-81. OCT4 is a nuclear marker specific for the ES cells whereas TRA-1-81 is a cell membrane marker expressed only on the human ES cells.

Summarizing, we were able to confirm that our newly established hES cell lines expressing the *MLL-AF9* fusion gene and the *FLT3-WT*/*FLT3-ITD* genes maintained their state of pluripotency, which was crucial for the use of these cell lines in our further experiments.

4.1.4. CHROMOSOMAL STABILITY OF NEW TRANSGENIC hES CELL LINES

Another very important aspect when working with human ES cells is the control of their chromosomal stability. Cultured hESC lines are an invaluable resource because they provide a uniform and stable genetic system for functional analyses and therapeutic applications. Nevertheless, these dividing cells, like other cells, probably undergo spontaneous mutation at a rate of 10^{-9} per nucleotide. Because each mutant

has only a few progeny, the overall biological properties of the cell culture are not altered unless a mutation provides a survival or growth advantage. Clonal evolution that leads to emergence of a dominant mutant genotype may potentially affect cellular phenotype as well. The observation that hESC lines maintained in vitro develop genetic and epigenetic alterations implies that periodic monitoring of these lines is required before they are used in *in vivo* applications and that some late-passage hESC lines may be unusable for therapeutic purposes (Maitra, Arking et al. 2005).

It our study, the genomic stability of hESC lines was monitored regularly by karyotype analysis (Table 4-1).

Table 4-1 | Control of the chromosomic stability of the newly established hES cell lines.

Genotype	Cell passages			
	Passage 0	**Early passages**	**Intermediate passages**	**Late passages**
AND2	46,XX	48,XX,+12,+20	48,XX,+12,+20	48,XX,+12,+20
AND2-NEO	-	48,XX,+12,+20	48,XX,+12,+20	48,XX,+12,+20
AND2-*MLL-AF9*	-	48,XX,+12,+20	48,XX,+12,+20	48,XX,+12,+20
AND2-NEO-*FLT3-WT*	-	48,XX,der(10) t(10;?)(p21;?),+12,+20,	48,XX,der(10) t(10;?)(p21;?),+12,+20	N.D.
AND2-NEO-*FLT3-ITD*	-	48,XX,der(10) t(10;?)(p21;?),+12,+20	48,XX,der(10) t(10;?)(p21;?),+12,+20	N.D.
AND2-*MLL-AF9-FLT3-WT*	-	48,XX,der(10) t(10;?)(p21;?),+12,+20	48,XX,der(10) t(10;?)(p21;?),+12,+20	N.D.
AND2-*MLL-AF9-FLT3-ITD*	-	48,XX,der(10) t(10;?)(p21;?),+12,+20	48,XX,der(10) t(10;?)(p21;?),+12,+20	N.D.

ND: not determined

We observed that the control hES cell lines and the *MLL-AF9* hES cell line acquired a non-significant chromosomal changes (the trisomy of the chromosome 12 and the chromosome 20) and these changes were maintained during their culture until late passages without accumulating of new rearrangements (Figures 4-14a-d).

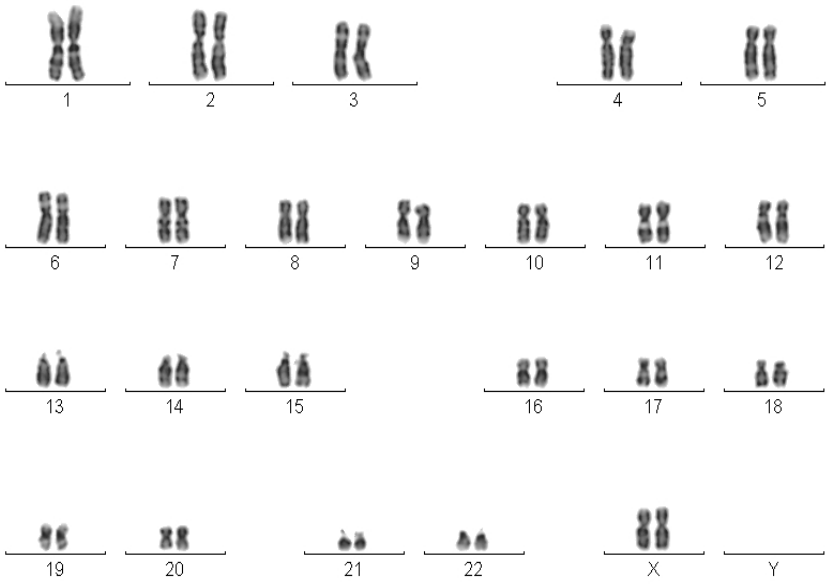

Figure 4-14a | Normal karyotype of the AND2 cell line at passage 6 (46,XX).

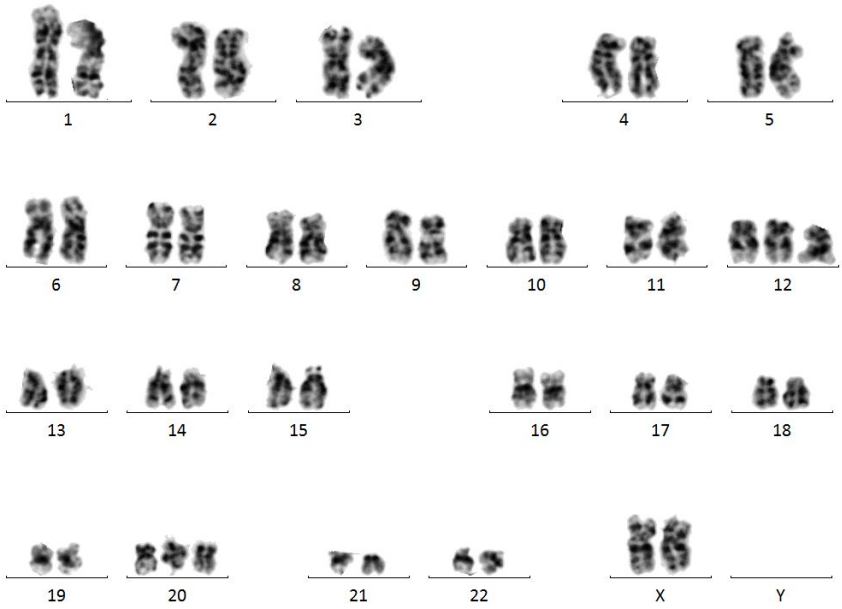

Figure 4-14b | Karyotype of the AND2 cell line at the passage 77 (48,XX,+12,+20) with trisomies of the chromosomes 12 and 20.

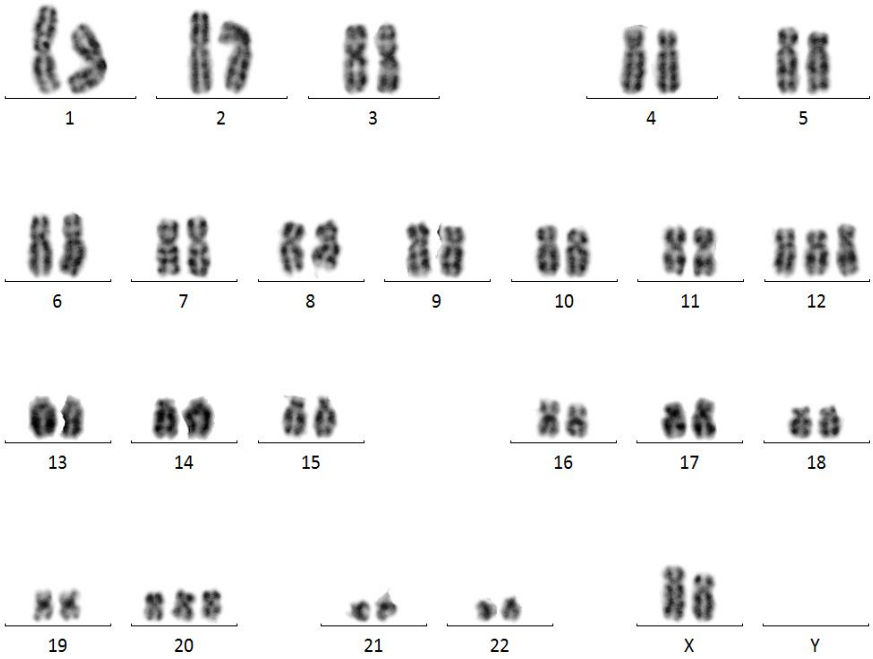

Figure 4-14c | Karyotype of the AND2-NEO cell line at passage 37 (48,XX,+12,+20) with trisomies of the chromosomes 12 and 20.

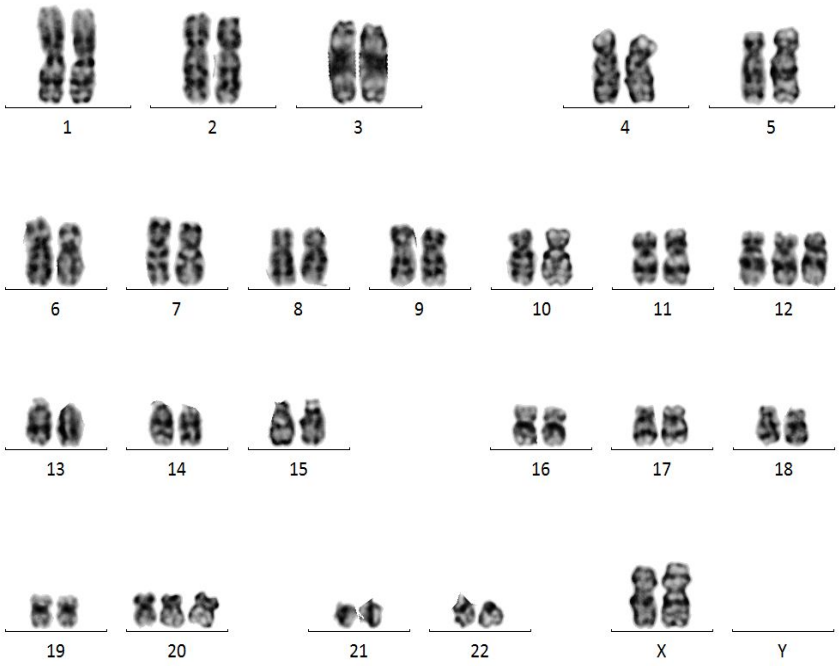

Figure 4-14d | Karyotype of the AND2-MLL-AF9 cell line at passage 18 (48,XX,+12,+20) with trisomies of the chromosomes 12 and 20.

In the case of the *FLT3*-expressing hES cell lines, we observed that the introduction and overexpression of both *FLT3-WT* and *FLT3-ITD* forms might lead to chromosomal rearrangements. Analysis of the karyotypes of our *FLT3*-expressing hES cell lines showed the presence of derivative of chromosome 10 (Figure 4-14e-h).

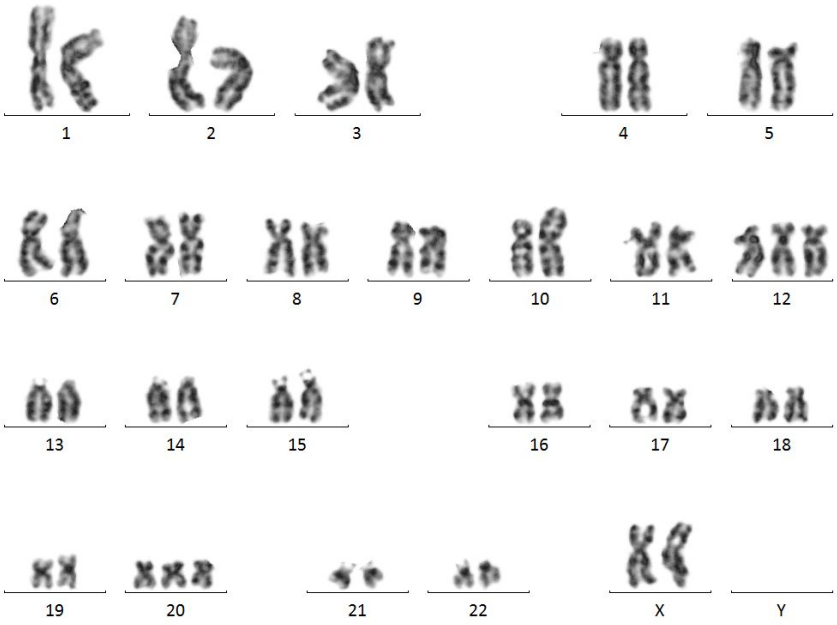

Figure 4-14e│Karyotype of the AND2-NEO-FLT3-WT cell line at passage 21 (48,XX,der(10)t(10;?)(p21;?),+12,+20) with trisomies of the chromosomes 12 and 20 and the derivative of the chromosome 10.

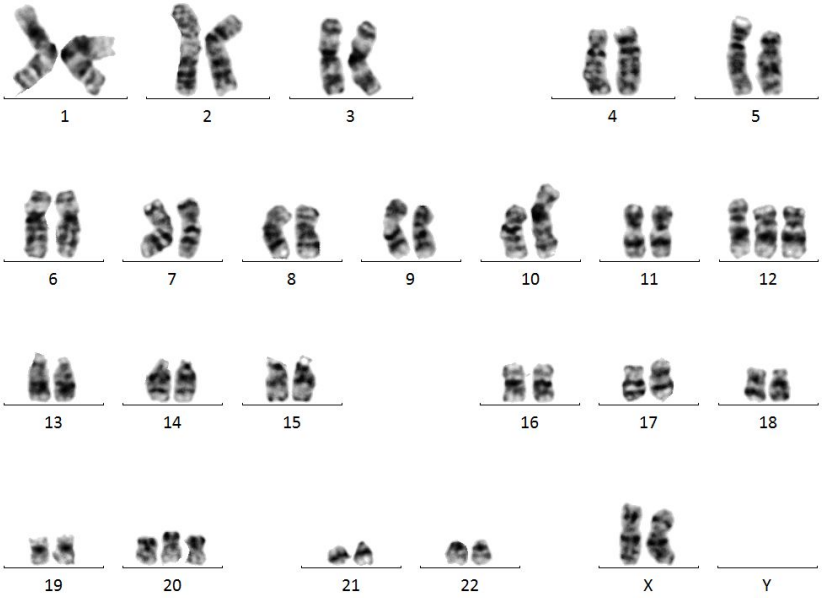

Figure 4-14f│Karyotype of the AND2-NEO-FLT3-ITD cell line at passage 16 (48,XX,der(10)t(10;?)(p21;?),+12,+20) with trisomies of the chromosomes 12 and 20 and the derivative of the chromosome 10.

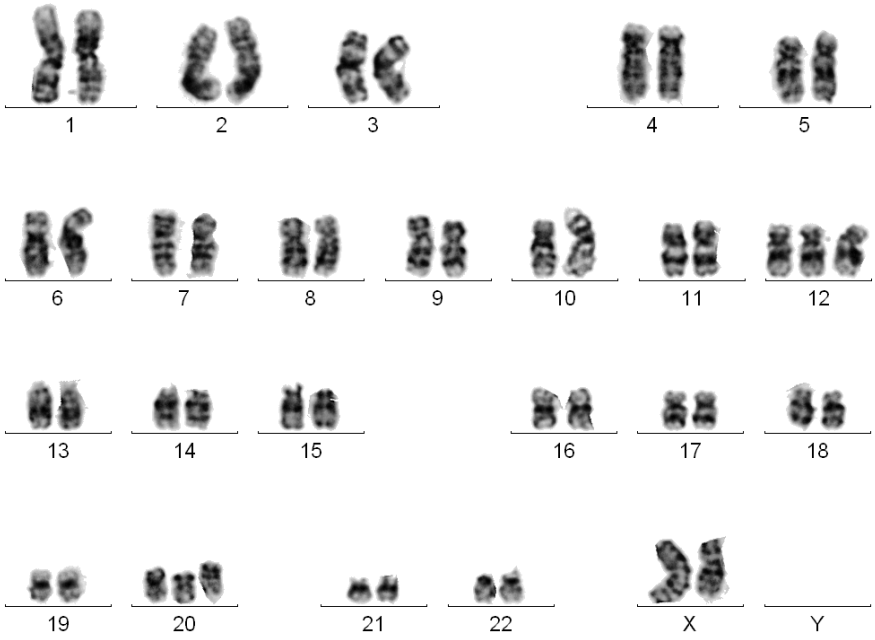

Figure 4-14g | Karyotype of the AND2-MLL-AF9-FLT3-WT cell line at passage 18 (48,XX,der(10)t(10;?)(p21;?),+12,+20) with trisomies of the chromosomes 12 and 20 and the derivative of the chromosome 10.

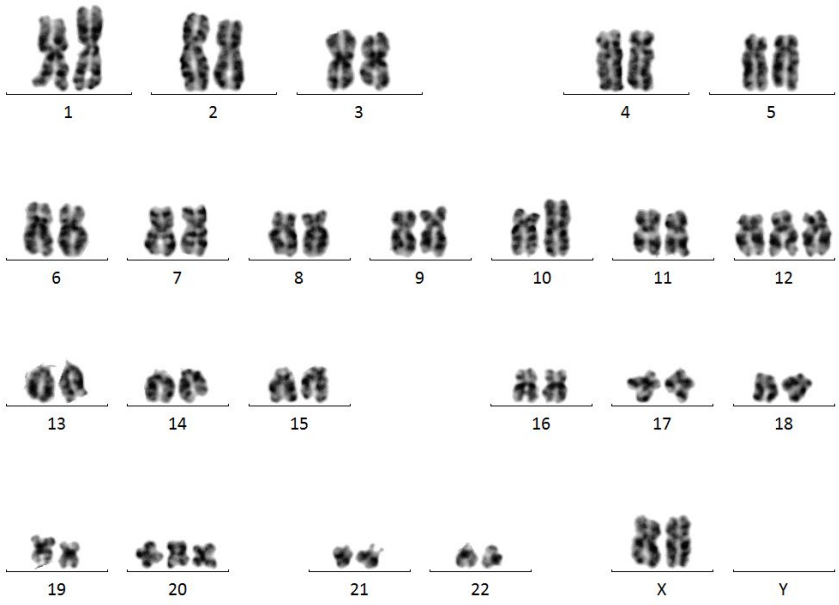

Figure 4-14h | Karyotype of the AND2-MLL-AF9-FLT3-ITD cell line at passage 22 (48,XX,der(10)t(10;?)(p21;?),+12,+20) with trisomies of the chromosomes 12 and 20 and the derivative of the chromosome 10.

4.1.5. CELL CYCLE ANALYSIS

To further characterize our newly established MLL-AF9/FLT3 hES cell lines, we decided to analyze the cell cycle of these hES cells and to control whether their genetic modifications (by lentiviral transduction) may deregulate this essential biological feature of the cell.

Embryonic stem cells show unusual cell-cycle features: they proliferate more rapidly than other cell types; the duration of the S phase is comparable to somatic cells but they have remarkably short G1 and G2 phases (Savatier, Lapillonne et al. 2002; Fujii-Yamamoto, Kim et al. 2005; White, Stead et al. 2005; Ballabeni, Park et al. 2011).

It has been shown, that one of the main features of *MLL*-rearranged leukemias is a blockage of the hematopoietic differentiation and am increased cellular proliferation. Moreover, the constitutive activation of the FLT3 receptor induced by the *FLT3-ITD* promotes the ligand-independent proliferation and also has been shown to block myeloid differentiation of early hematopoietic cells in mouse model systems (Kiyoi, Towatari et al. 1998; Hayakawa, Towatari et al. 2000; Kiyoi, Ohno et al. 2002; Zheng, Friedman et al. 2002).

In our study, we addressed the question whether the expression of the *MLL-AF9* and the *FLT3* genes could provide any proliferative advantage to undifferentiated hESC cultures. To address this, cell cycle distribution was analyzed by measuring IP incorporation.

As presented on Figures 4-15a-b, no differences in the proportion of cycling cells were observed between all hES cell lines. Unexpectedly, neither *MLL-AF9* nor *FLT3-WT/ITD* expression conferred any survival advantage to undifferentiated hESC cultures. Similar results reported recently Bueno et al. (Bueno, Montes et al. 2012), after expressing another fusion gene *MLL-AF4* in hES cells. They also did not observe any positive upregulation of cell proliferation in their *MLL-AF4* hESC model.

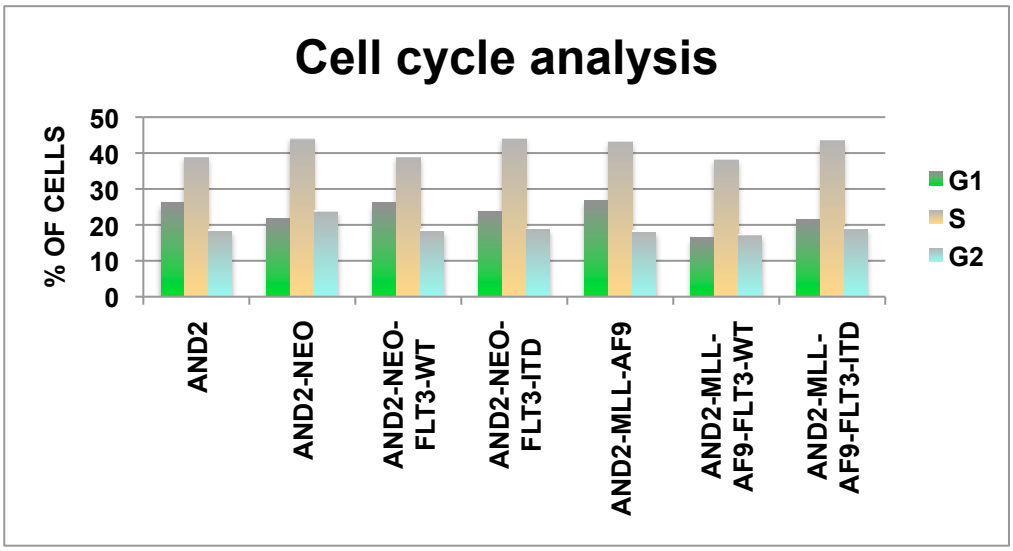

Figure 4-15a | Readout of the cell cycle analysis in the control hES cell line and the newly established MLL-AF9/FLT3 hES cell lines.

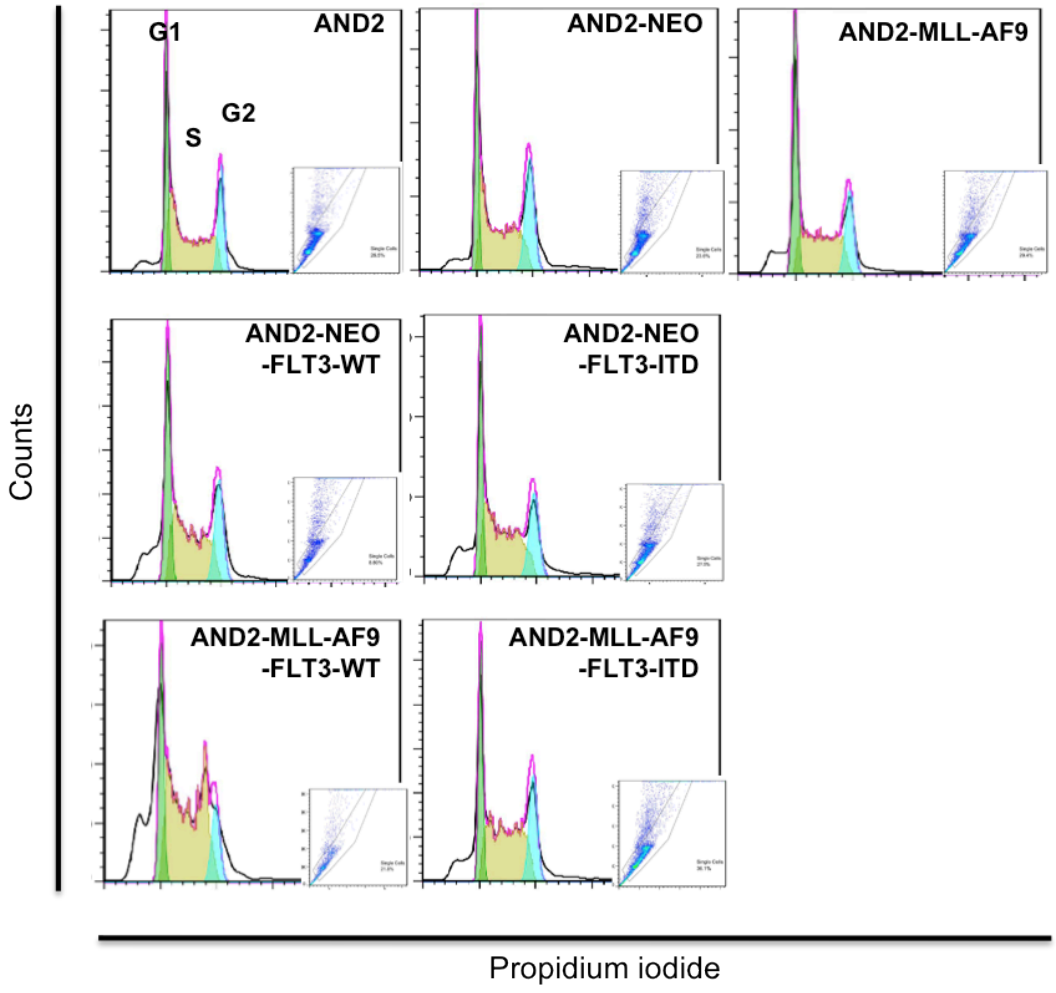

Figure 4-15b | Distribution of the cell cycle in the control hES cell line and the newly established MLL-AF9/FLT3 hES cell lines.

4.2. HEMATOPOIESIS-DIRECTED DIFFERENTIATION OF TRANSGENIC hESC LINES

Establishment of pluripotent human embryonic stem cell lines (hESC) (Thomson, Itskovitz-Eldor et al. 1998) provides a unique opportunity to investigate the early developmental processes of cell fate specification. The removal of hESCs from culture conditions containing basic fibroblast growth factor (bFGF, a cytokine that inhibits their spontaneous differentiation) results in the aggregation of hESCs into clusters of cells, termed human embryonic bodies (hEBs), that are of capable of differentiation into all primary germ layers (Schuldiner, Yanuka et al. 2000).

The study of human hematopoiesis using hESCs has been one of the most successful fields to date. It is known that hESCs, characterized by their pluripotency and theoretically unlimited proliferation ability, are capable of differentiate into all (almost but nor entirely functioning) blood cell types under suitable *in vitro* culture conditions (Kaufman, Hanson et al. 2001; Menendez, Wang et al. 2004; Vodyanik, Bork et al. 2005; Woll, Martin et al. 2005; Galic, Kitchen et al. 2006; Qiu, Olivier et al. 2008). Various studies have shown that hESC differentiation into hematopoietic lineages closely mimics the embryonic hematopoiesis, making them an incomparable tool for the study of hematopoiesis during embryonic development in states of health or disease (Lerou and Daley 2005; Lensch and Daley 2006; Menendez, Bueno et al. 2006).

Using the hEB model of differentiation it has been shown (Wang, Menendez et al. 2005) that human ESC-derived hematopoietic cells emerge from a cellular subset of the embryonic endothelium expressing CD31 (PECAM-1), Flk-1, and VE-Cadherin but lacking CD45 ($CD45^{-}CD31^{+}$, frequently named as hemogenic precursors) (Ramos-Mejia, Melen et al. 2010). These hemogenic precursors are the exclusively responsible for hematopoietic potential of differentiated hESCs.

Taking advantage of these discoveries and the ability of hES cells to differentiate into hematopoietic lineages and to recapitulate the process of early hematopoiesis, we decided to use this model to investigate the effects of the *MLL-AF9* fusion gene and the *FLT3-ITD* mutation on the onset of hematopoiesis. Basing on previous results and in our own experience, we decided to use the hEB system to differentiate hES cells into hematopoietic cells. Examples of human EB created for the purpose of this study are depicted on Figure 4-16.

It is important to highlight that all hESC lines (wild type and transgenic) used in this study showed the ability to form embryonic bodies, which means that the genetic manipulation did not affect their stem cell properties.

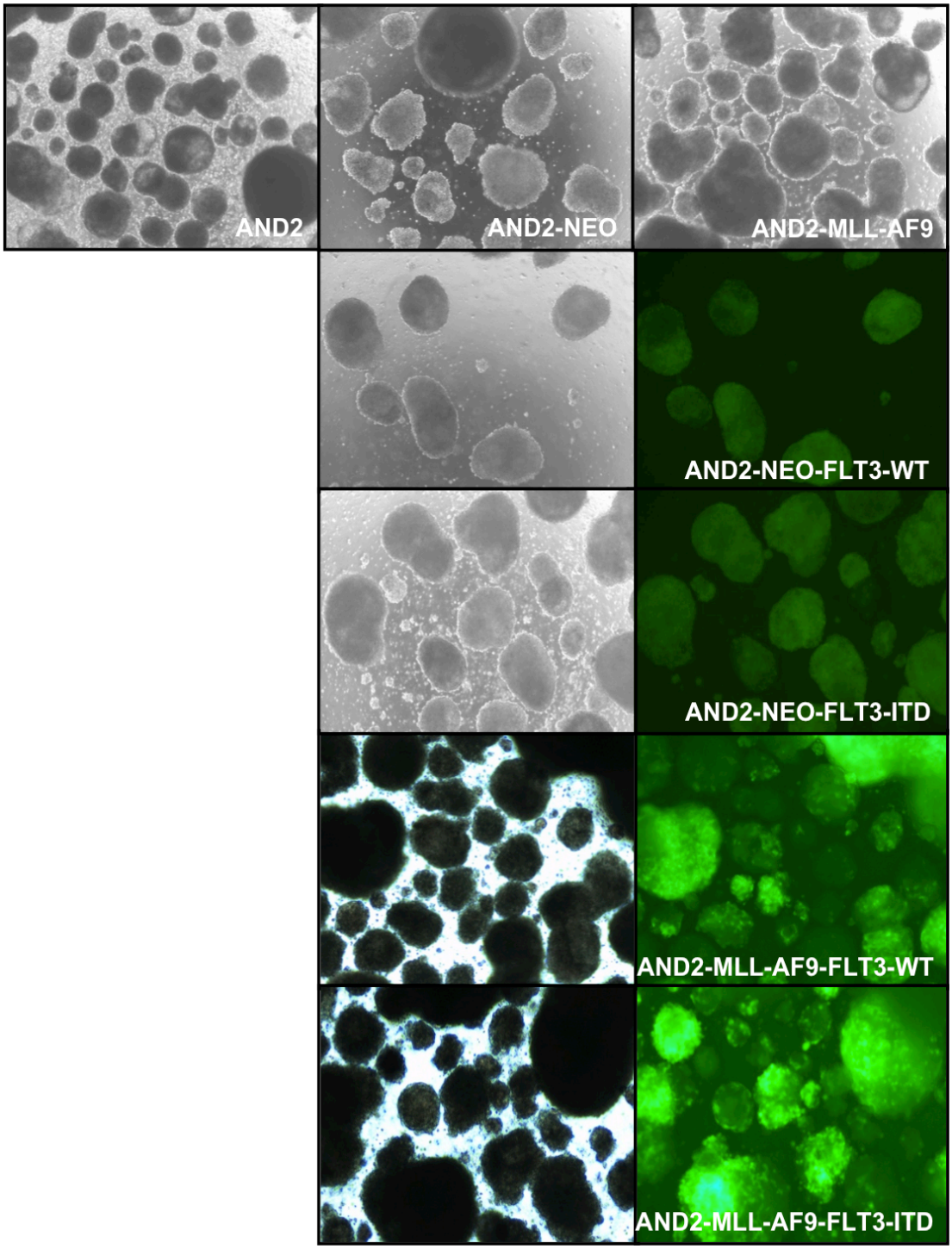

Figure 4-16 | Morphology of the Human Embryonic Bodies (hEBs). No differences in the ability of EB formation were observed between all transgenic hES cell lines.

4.2.1. *MLL-A9* EXPRESSION AND ITS IMPACT ON HEMATOPOIETIC DIFFERENTIATION OF hESCs

As the main objective of this study, we decided to investigate first the effect of leukemic fusion *MLL-AF9* on the process of hematopoietic differentiation of hES cells. During human embryonic body (hEB) differentiation, a population of primitive hemogenic precursors arises, which is uniquely responsible for hematopoietic and endothelial development (Wang, Li et al. 2004; Ramos-Mejia, Melen et al. 2010).

We were successful in establishing the hematopoietic differentiation using the hEB protocol. In our hands, both the wild type hES cell line AND2 and the control cell line AND2-NEO, showed a typical hematopoietic differentiation dynamics with the first hemogenic precursors ($CD45^-CD34^+CD31^+$, green line) arising between days 7 and 10. Starting from day 10, primitive hematopoietic progenitors ($CD45^+CD34^+CD31^-$, blue line) appeared and a subsequent differentiation into mature blood cells ($CD45^+CD34^-CD31^-$, red line) was observed at day 15 (Figures 4-17a and b).

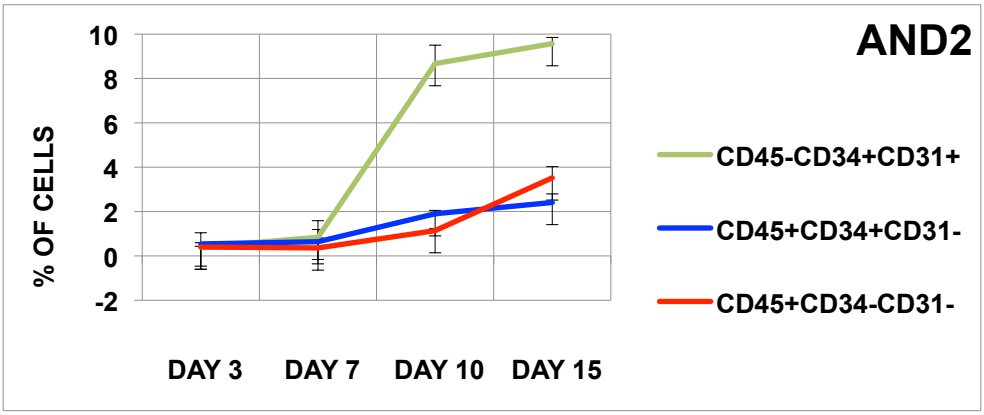

Figure 4-17a | Time course of hematopoietic differentiation of wild type hES cell line AND2. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

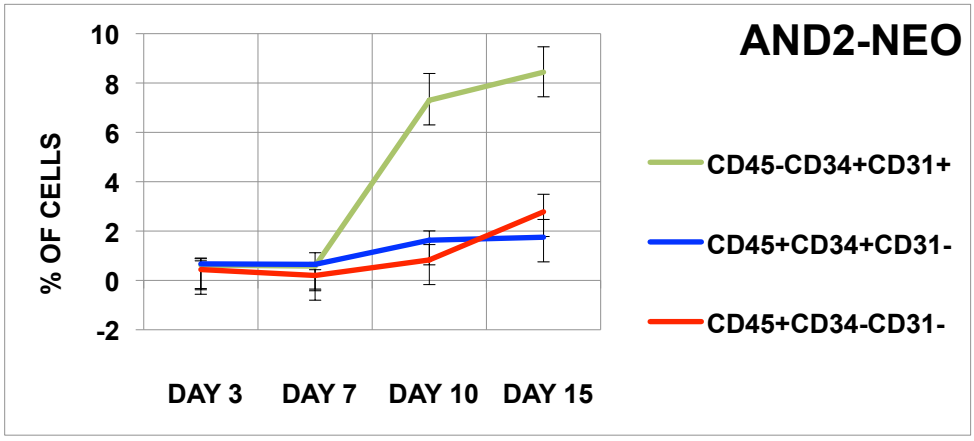

Figure 4-17b | Time course of hematopoietic differentiation of the control hES cell line AND2-NEO. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

These results indicated that the genetic introduction of a lentiviral vector did not affect the differentiation capacity of hESC. Then, we proceeded to the next step, the introduction of oncogenes.

In the case of the the *MLL-AF9* expressing hES cell line, the differentiation into hemogenic precurosrs was maintained, but on a slightly descreased level when compared with the hES control cell lines (5% versus 9-10%, respectively). Subsequent differentiation of *MLL-AF9* hemogenic precursors into primitive blood cells and mature blood cells was completely blocked (from 2% to 0%) (Figure 4-17c).

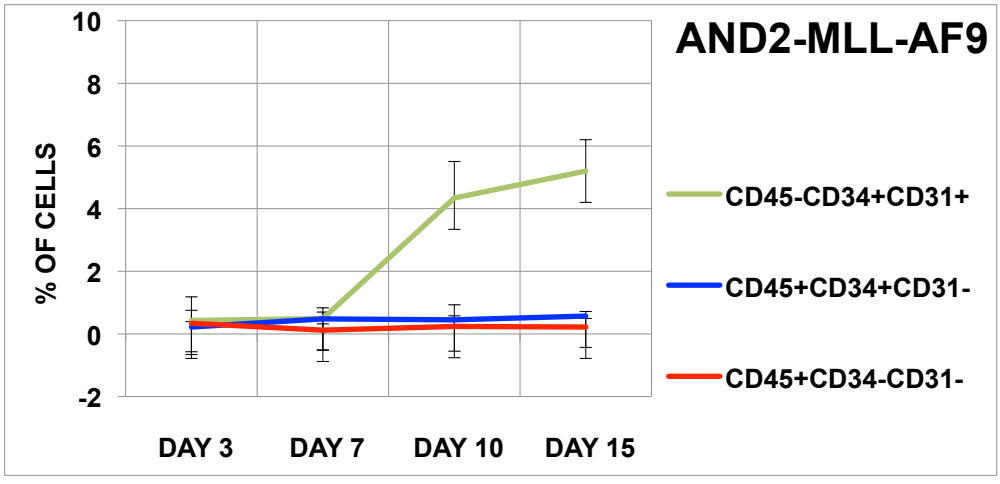

Figure 4-17c | Time course of hematopoietic differentiation of the MLL-AF9 hES cell line AND2-MLL-AF9. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

In summary, the expression of the *MLL-AF9* fusion gene in hESC abrogated the hematopoietic differentiation as reflected by a robust reduction on the frequency of both $CD45^{+}CD34^{+}$ (2.5- to 4.2-fold decrease) and total $CD45^{+}$ blood cells (4.75- to 16-fold descrease) at day 10 and day 15 of hEB development (Figure 4-17d).

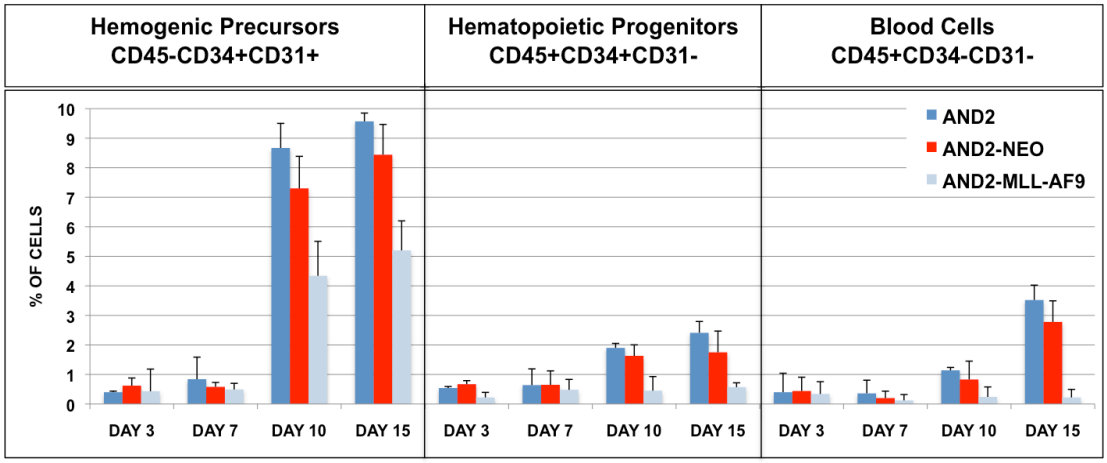

Figure 4-17d | Comparision of the hematopoietic differenitation efficiencies between the control hES cell lines and the MLL-AF9 hES cell line. The plotted results are the median of three independent experiments ± SEM.

4.2.2. *FLT3* AS A POSITIVE REGULATOR OF HEMATOPOIETIC DIFFERENTIATION

FLT3, a member of the receptor tyrosine kinase (RTK) class III, is preferentially expressed on the surface of a high proportion of acute myeloid leukemia (AML) and B-lineage acute lymphocytic leukemia (ALL). An interaction of FLT3 and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal hematopoietic cells but also leukemia cells (Ono, Nakajima et al. 2005; Zheng and Small 2005; Levis 2011; Godfrey, Arora et al. 2012).

In this study, we decided to investigate the possible effect of ectopic expression of *FLT3-WT* and its mutant *FLT3-ITD*, on the process of hematopoietic differentiation from hESCs (Figures 4-17e-f). In the case of *FLT3-WT* overexpression, there was no visible effect on the levels of hemogenic precursors (green line) but we observed a significantly increased level of hematopoietic progenitors (blue line). Moreover, we could also observe that these hematopoietic progenitors started to appear much earlier (at day 7). There was no visible effect on the percentage of mature blood cells, when comparing to wild type hES cell line (Figure 4-14e).

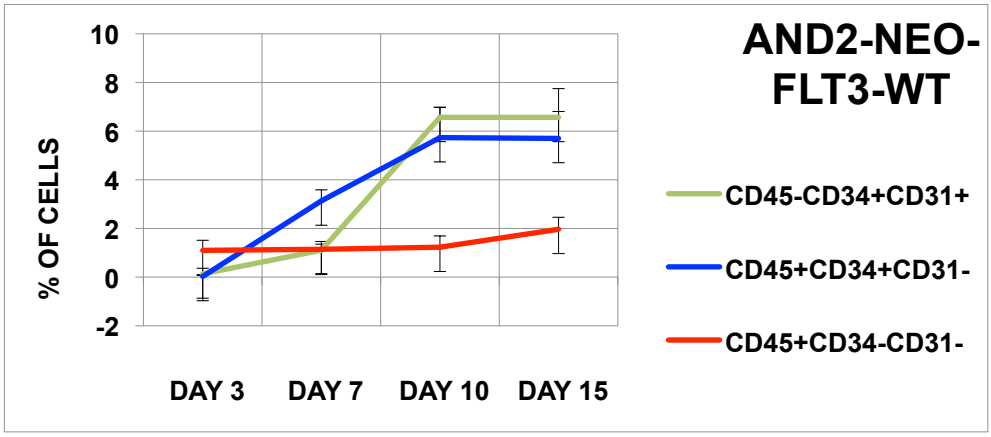

Figure 4-17e | Time course of hematopoietic differentiation of the FLT3 hES cell line AND2-FLT3-WT. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

In the case of *FLT3-ITD* overexpression, the dynamic of hematopoietic differentiation was maintained, but at descreased levels. From the 9-10% of hemogenic precursors observed in the hES control cell lines (Figures 4-17a-b) to the 4% found in this model (Figure 4-17f). The maximum percentage of both hemogenic precursors and hematopoeitic progenitors was observed at day 10 with a downward tendency at day 15. The percentages of mature blood cells were maintained at very lov level, as for the other models.

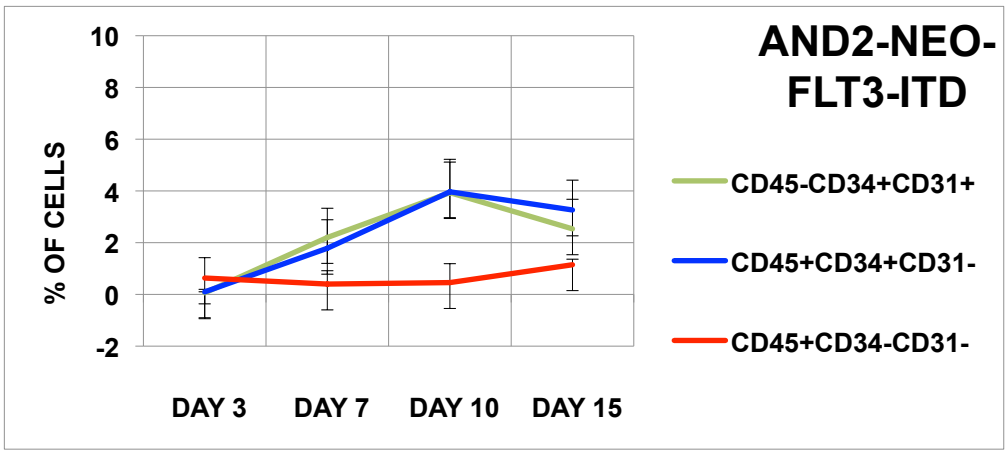

Figure 4-17f | Time course of hematopoietic differentiation of the FLT3 hES cell line AND2-FLT3-ITD. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

Summarizing, although the overexpression of *FLT3* genes slightly reduced the number of hemogenic precursors, we dobserved an increased numbers of hematopoietic precursos appearing earlier in the timecourse of hematopoeitic differentiation. This probably may be due to the accelerating activity of the *FLT3* gene. In detail, the overexpression of *FLT3* definitely improved hematopoietic differentiation as reflected by increase on the frequency of $CD45^{+}CD34^{+}$ cells (1.36- to 3-fold increase) at day 10 and day 15 of hEB development (Figure 4-17g). Also, the apprearence of hematopoietic progenitors ($CD45^{+}CD34^{+}CD31^{-}$) seemed to be accelerated, as reflceted by increase on their fenquency (2.8- to 4.9-fold increase) at day 7 of hEB development (Figure 4-17g). From this experiment we can conclude that *FLT3* sirves as a positive regulator of hematopoietic differentiation, when overexpressed in wild type hES cells.

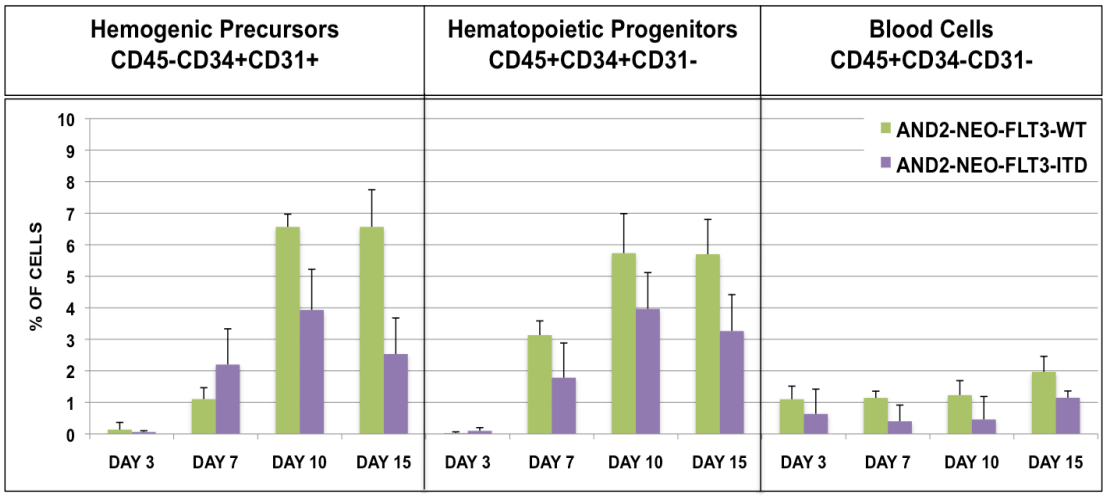

Figure 4-17g | Comparision of the hematopoietic differenitation efficiencies of the two FLT3 hES cell lines: AND2-FLT3-WT and AND2-FLT3-ITD. The plotted results are the median of three independent experiments ± SEM.

4.2.3. *FLT3-ITD* AS A SECONDARY LEUKEMOGENIC HIT TO *MLL-AF9*

The mutated form of the *FLT3* gene, *FLT3-ITD*, is found in approximately 30% of adult AML patients and in approximately 10% of childhood AML patients (Stubbs, Kim et al. 2008). In this study, it has been investigated possible impact of this mutation and the wild type *FLT3* on the process of hematopoietic differentiation, by ectopically expressing the *FTL3-ITD* in the *MLL-AF9* hES cells. In the case of overexpression of the *FLT3-WT* form in *MLL-AF9* hES cell line, only very low levels of hemogenic precursors and hematopoietic progenitors were observed, confirming the inhibiting effect of *MLL-AF9* expression. As expected, there was no signs of differentiation into mature blood cells (Figure 4-17h, see also Figures 4-17c and d for comparison).

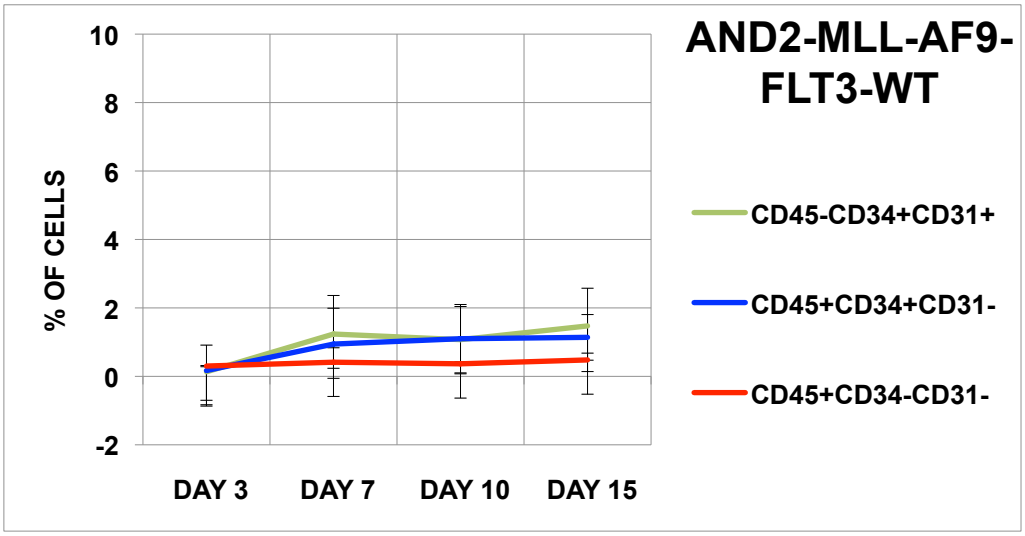

Figure 4-17h | Time course of hematopoietic differentiation of the MLL-AF9-FLT3 hES cell line AND2-MLL-AF9-FLT3-WT. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

After the co-expression of the leukemic *MLL-AF9* fusion gene and the *FLT3-ITD* mutant form, the hematopoietic potential of hES cell line AND2 was completely abrogated (Figure 4-17i).

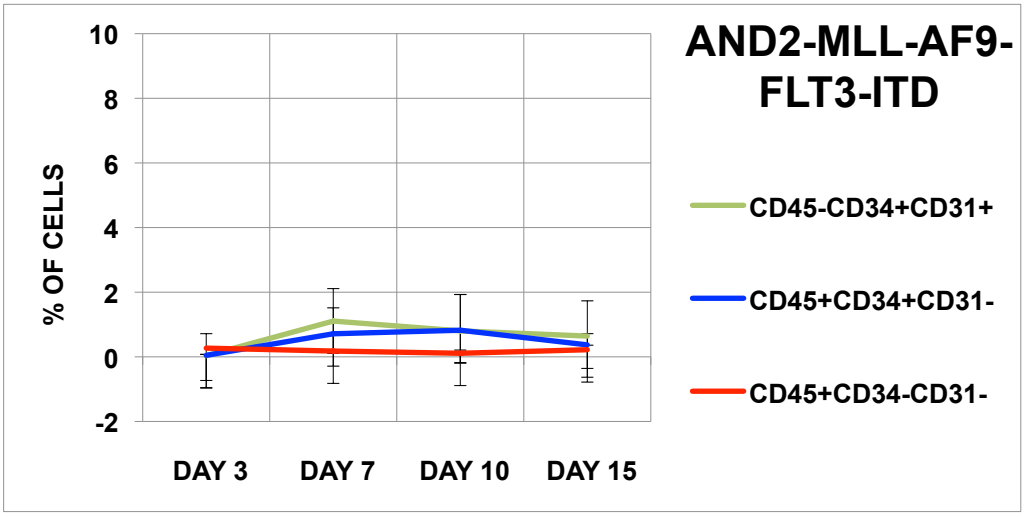

Figure 4-17i | Time course of hematopoietic differentiation of the MLL-AF9-FLT3 hES cell line AND2-MLL-AF9-FLT3-ITD. The proportion of the different lineages is shown during the hematopoietic differentiation experiment. The plotted results are the median of three independent experiments ± SEM. Red line ($CD45^+CD34^-CD31^-$): mature blood cells; Blue line ($CD45^+CD34^+CD31^-$): primitive hematopoietic precursors; Green line ($CD45^-CD34^+CD31^+$): hemogenic precursors.

There were no signs of hemogenic or hematopoietic specification as reflected by very low frequency of $CD45^-CD34^+CD31^+$ and $CD45^+CD34^+CD31^-$ at day 10 and day 15 of hEB development, respectively (Figure 4-17j).

In summary, our findings suggested that the simultaneous overexpression of the *FLT3* gene (both the WT and the ITD forms) and the *MLL-AF9* fusion gene results in a complete abrogation of the hematopoietic differentiation potential of hES cells.

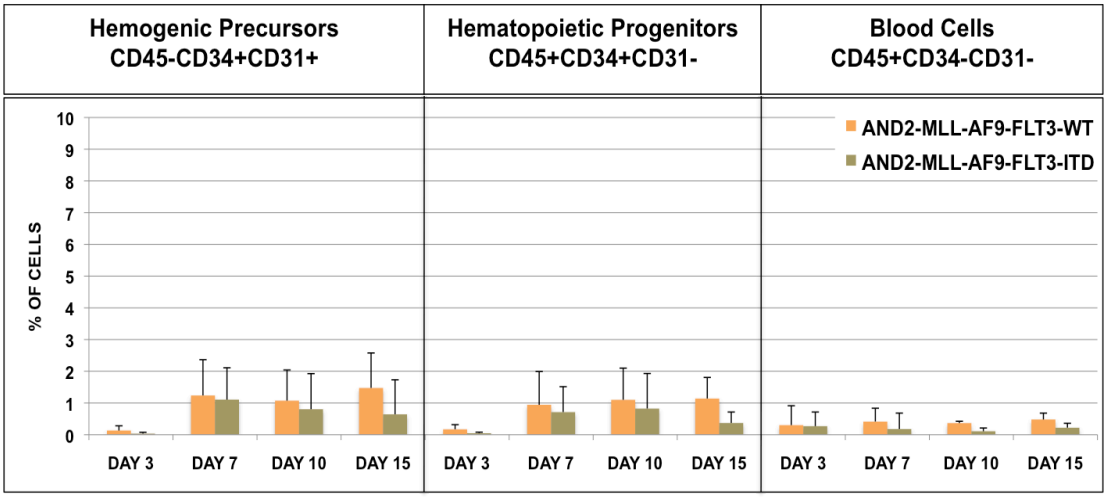

Figure 4-17j | Comparision of the hematopoietic differenitation efficiencies of the two MLL-AF9-FLT3 hES cell lines: AND2-MLL-AF9-FLT3-WT and AND2-MLL-AF9-FLT3-ITD. The plotted results are the median of three independent experiments ± SEM.

4.3. COLONY FORMING ASSAY

Hematopoiesis is the process by which stem cells divide and differentiate to produce the multiple types of mature cells found in blood. The process begins in early embryonic development and continues throughout adult life, primarily in the bone marrow. Various *in vivo* and *in vitro* assays have been developed to detect and assess the functionality of the stem cells and early multi-potential progenitors. Among them, colony-forming cell (CFC) assays may be used to quantify lineage-restricted progenitors in a simple in vitro assay. When cultured in a semi-solid medium containing the appropriate cytokines, CFCs are able to divide and differentiate into a colony of more mature cells that can be detected by light microscopy. This allows for the quantification of erythroid, myeloid, lymphoid, megakaryocytic, and multi-potential cell lineages from various cell sources.

The cells that form hematopoietic colonies (so-called CFUs or CFCs) expressing the $CD34^+$ marker represent a stage of hematopoietic differentiation between hematopoietic stem cells (HSCs) and more terminally differentiated cells (such as erythrocytes, granulocytes, monocytes, or platelets). In our study, these $CD34^+$ CFCs were purified (Table 4-2) from hEB cultures (Figure 4-16) and then identified by culturing them in a semisolid media (Methylcellulose) supplemented with a cocktail of cytokines that promote the localized expansion and differentiation of hematopoietic cells in discrete colonies (Figure 4-19).

Table 4-2 | A sample of the separation of the $CD34^+$ cells at day 15 of hEB differentiation.

Cell line	Total cell number	$CD34^+$ fraction	% of $CD34^+$ cells
AND2	12.75×10^4	11.4×10^3	8.94
AND2-NEO	13.21×10^4	10.4×10^3	7.89
AND2-NEO-FLT3-WT	12.13×10^4	13.2×10^3	10.88
AND2-NEO-FLT3-ITD	14.86×10^4	9.2×10^3	6.23
AND2-MLL-AF9	12.56×10^4	5.2×10^3	4.21
AND2-MLL-AF9-FLT3-WT	19.52×10^4	2.5×10^3	1.28
AND2-MLL-AF9-FLT3-ITD	18.91×10^4	1.7×10^3	0.90

It is commonly known that leukemia is a process characterized by the accumulation of immature hematopoietic precursors, which are not able to differentiate into mature blood cells. In this pathological situation, the differentiation and clonogenic potential of these precursors is compromised. Recently, Bueno et al., (Bueno, Montes et al. 2012) showed that the expression of the *MLL-AF4* fusion gene in hES cells not only blocks their hematopoietic differentiation but also decreases significantly their clonogenic potential and the ability to form colonies of mature blood cells.

In our study (chapter 4.2) we have shown that the hematopoiesis-directed differentiation of hES cells is blocked by the expression of the *MLL-AF9* fusion (i.e. the generation of $CD45^{+}$ blood is abrogated). We wondered whether this fusion gene has also influence on the clonogenic potential of hematopoietic progenitors derived from hES cells. As we can observe on the Figure 4-18, the expression of the *MLL-AF9* fusion gene in hES cells strongly compromised the clonogenic potential of hematopoietic progenitors derived from day 15 of hEB culture (Figure 4-18).

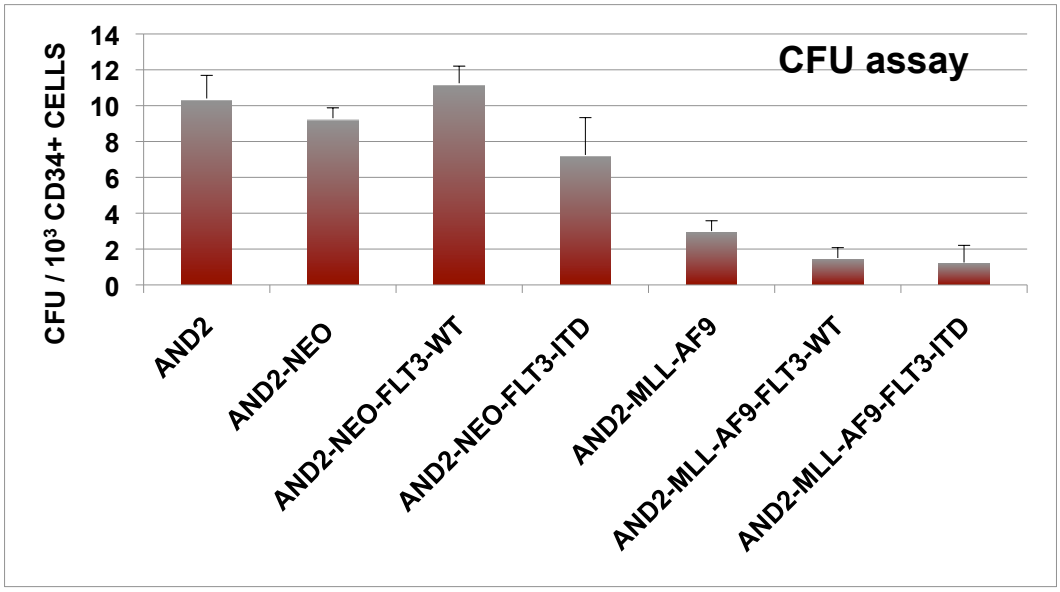

Figure 4-18 | Colony Forming Unit (CFU) readout obtained from CD34+ cells purified at day 15 during the hEB differentiation process. The plotted results are the median from three independent experiment ± SEM.

The morphology of observed colonies was typical for myeloid lineages (macrophage and granulocyte-macrophage) (Figure 4-19).

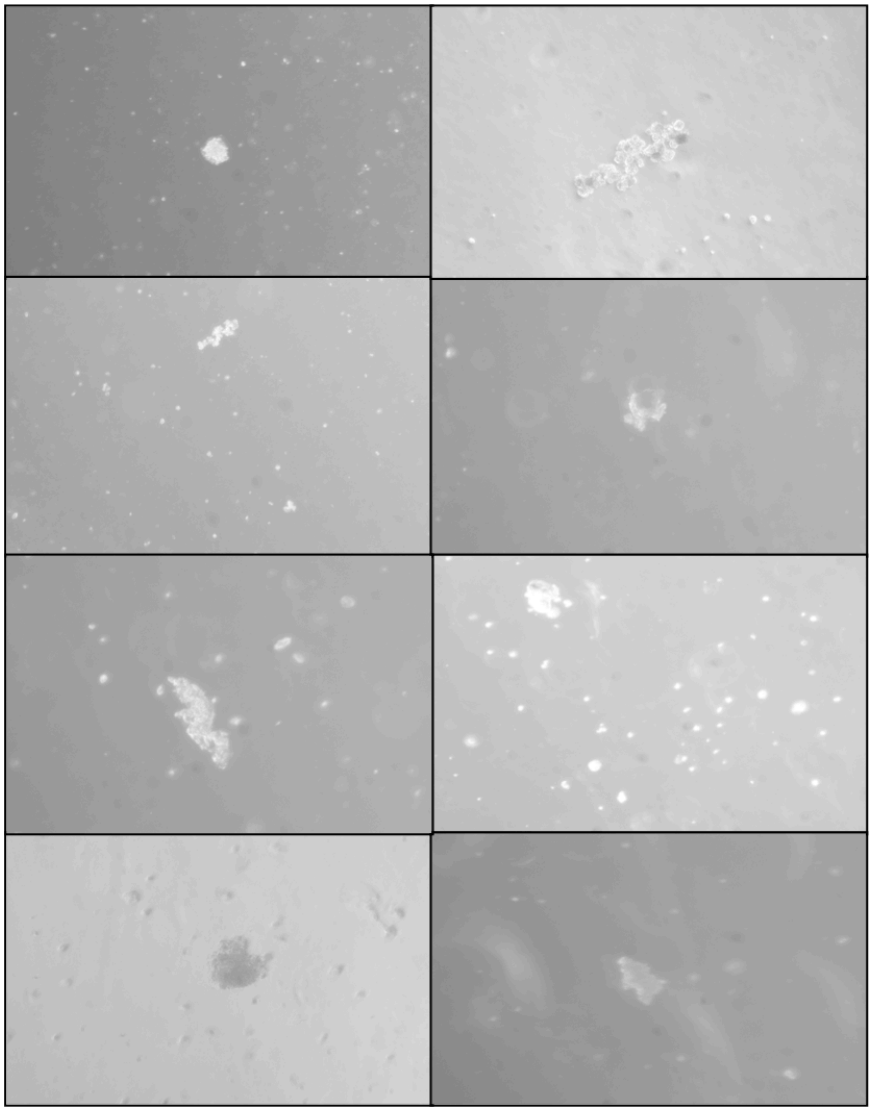

Figure 4-19 | Macrophage and granulocyte-macrophage morphology of observed hematopoietic colonies obtained in the CFU assay.

Concluding, hematopoiesis generated from both *MLL-AF9* and *MLL-AF9-FLT3* hESCs (see complete list in Table 4-2) displayed a highly reduced clonogenic potential measured by the ability to form CFUs in semisolid cultures (Figure 4-18). These data together with hematopoietic differentiation results (Figures 4-17a-j) indicate that *MLL-AF9* impairs the hemogenic differentiation of hES cells (decreased percentages of $CD45^{-}CD34^{+}CD31^{+}$ cells) and strongly compromise their subsequent hematopoietic differentiation and hematopoietic commitment (decreased percentages of $CD45^{+}CD34^{+}CD31^{-}$ and $CD45^{+}CD34^{-}CD31^{-}$ cells).

4.4. EXPRESSION OF POSSIBLE *MLL-AF9* TARGET GENES

It has been shown, that aberrant expression of *HOX* genes may contribute to leukemogenesis (Buske and Humphries 2000). Forced expression of *HOXA9* and *MEIS1* (HOX-binding partner) lead to the rapid development of myeloid leukemia in mice (Kroon, Krosl et al. 1998). It has been suggested that *HOXA9* and *MEIS1* suppress differentiation and maintain self-renewal of progenitor cells in response to growth factors (Calvo, Knoepfler et al. 2001).

It is also well known, that the expression of *HOX* genes (themselves acting as transcription factors) is positively regulated by the *MLL* gene (Buske and Humphries 2000; Ernst, Wang et al. 2002). Patients with acute leukemias carrying *MLL* rearrangements also show increased levels of selected *HOX* genes (Imamura, Morimoto et al. 2002; Ferrando, Armstrong et al. 2003).

The clustered homeobox or *HOX* genes encode transcription factors, which were first recognized as a playing mayor role in regulating embryonic cell fate of lower organisms (Gehring 1987; Kessel and Gruss 1990). In mammals, 39 different *HOX* genes are organized into four clusters (*HOX*A, B, C and D) located on four different chromosomes. In embryonic development their expression patterns are spatially and temporally restricted according to their 3´ to 5´ position on the chromosome (Graham, Papalopulu et al. 1989).

A number of studies have suggested a role of *HOX* genes in normal hematopoietic development (Lawrence and Largman 1992). For example, the patterns of expression of some members of the *HOX*A and B gene clusters in hematopoietic cells are closely related to their stage of differentiation. 5´ *HOX* genes such as *HOXA9* and A10 are highly expressed among $CD34^+$ cells, including the subpopulations enriched for both primitive and lineage-committed progenitors, and down-regulated in more differentiated $CD34^-$ cells. *HOX* genes with more 3´ locations, such as *HOX*B3 and B4, are down-regulated within the $CD34^+$ population as cells leave the most primitive cell compartment and express lineage commitment (Sauvageau, Lansdorp et al. 1994).

In this study, we decided to investigate whether the expression of *MLL-AF9* and *FLT3-WT*/*ITD* genes may have impact on the activation of clustered *HOX* genes in our hES cell models. The results of the quantitative analysis of the expression of selected possible *MLL-AF9* target genes are shown on Figure 4-20.

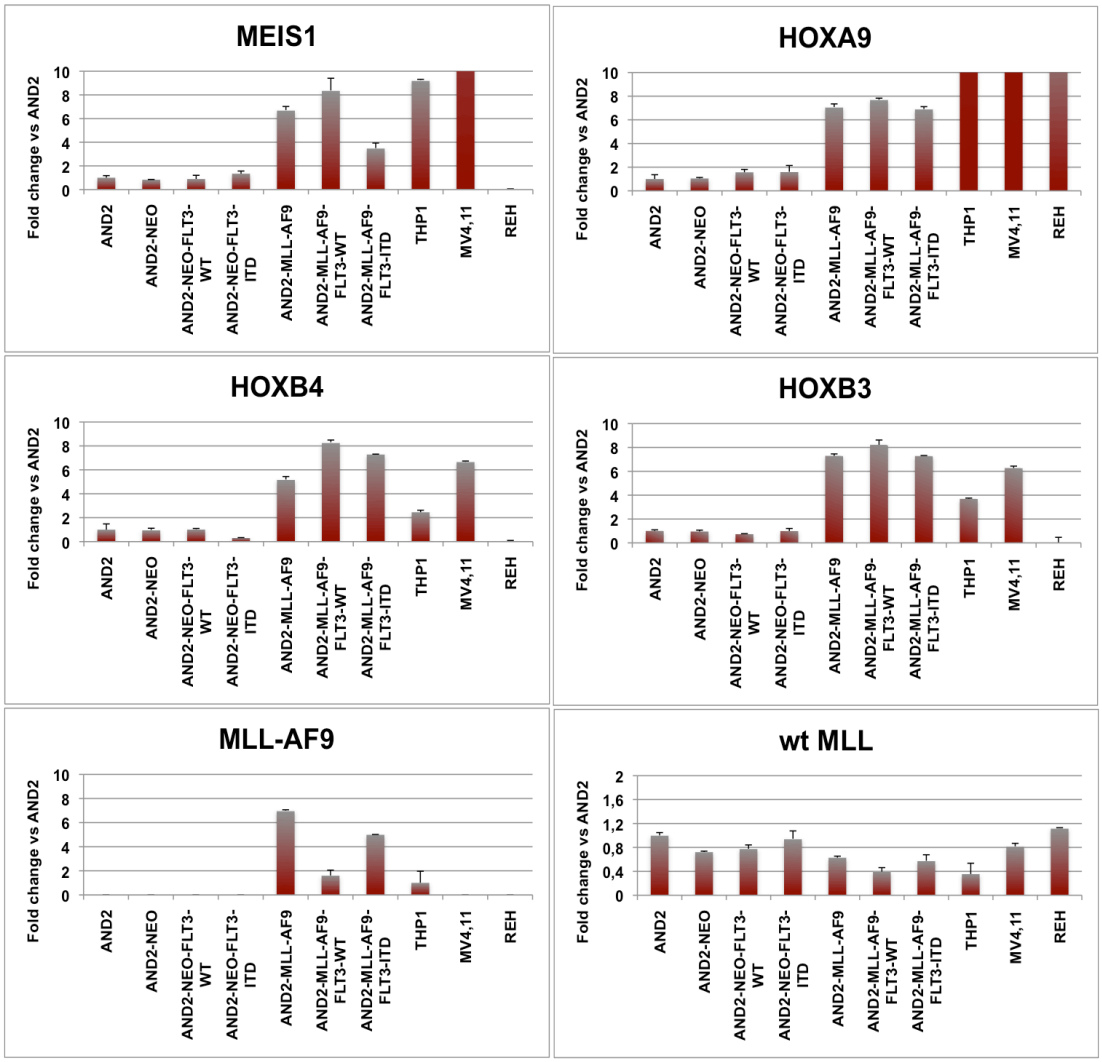

Figure 4-20 | Expression of selected *HOX* genes and *MLL* co-factor *MEIS1* in newly established transgenic hES cell lines measured by qPCR. The figure on the left bottom panel represents the confirmation of the *MLL-AF9* fusion gene expression in the transduced hES cell lines. Presented data are the median of three independent analysis ± SEM.

As we can observe, the expression of *FLT3-WT* and *FLT3-ITD* genes did not deregulate the levels of mRNA of selected genes (bars 3 and 4) when compared with the wild type cell line AND2 (bar 1). The level of endogenic WT *MLL* was also not affected by the expression of the oncogenes. Nevertheless, the expression of the *MLL-AF9* fusion gene in undifferentiated hES cells upregulated the mRNA levels of the *MEIS1* gene and also selected *HOX* genes. These results are in line with previous reports, which show that clustered *HOX* genes are upregulated in leukemia patients and this upregulation may be one of the mechanisms of *MLL-AF9*-driven leukemogenesis.

DISCUSSION

5.1. THE HUMAN ESC MODELS EXPRESSING THE *MLL-AF9* AND THE *FLT3-ITD* GENE

Leukemias harboring the *MLL-AF9* fusion gene are associated with a dismal prognosis and their clinical consequences are well characterized. *MLL-AF9*-expressing AML cells have morphologic and immunophenotypic features mostly consistent with a myelomonocytic differentiation. Patients with the *MLL-AF9* fusion gene have an intermediate to poor prognosis, suggesting that the *MLL-AF9* expression is associated with a more aggressive disease that includes resistance to chemotherapy (Schoch, Schnittger et al. 2003). *MLL* rearrangements arise in utero, are found in the majority of infants with acute leukemia, and are unique in their ability to produce overt clinical disease after only a few months (Ford, Ridge et al. 1993). Concerning the biological processes affected by this fusion gene a little is known and our understanding of its transformation capacities is limited. It is also not known at which stage of hematopoietic differentiation this translocation can occur and which is the critical one in the process of leukemiogenesis.

We have recently showed (Wei, Wunderlich et al. 2008) that the primary human hematopoietic progenitor cells (HPCs) can undergo leukemic transformation in response to the *MLL-AF9* chimeric oncogene in a manner that faithfully recapitulates many features of the clinical disease. Like *MLL-AF9* patient samples, the normal cord blood cells retrovirally transduced with the *MLL-AF9* fusion gene displayed essentially unlimited replicative potential, have myelomonocytic or pro-B cell features, and were leukemogenic in mice. Moreover, the transcriptome of these experimentally created cell lines extensively parallels that of primary leukemia cells form AML patients. These results suggested that the expression of the *MLL-AF9* fusion gene is the primary molecular defect responsible for the defining characteristics of acute myeloid leukemia. Moreover, these data support the hypothesis, based on the clinical observations, that *MLL* fusion genes require fewer independent oncogenic events for leukemic transformation than other fusion oncoproteins. *MLL* fusions are associated with the shortest latency periods documented between the appearance of a karyotypic abnormality and the clinical manifestation of malignancy.

FLT3, a member of the receptor tyrosine kinase (RTK) class III familiy of proteins, is preferentially expressed on the surface of hematopoietic precursors in a high proportion of acute myeloid leukemia (AML) and B-lineage acute lymphocytic leukemia (ALL). An interaction of *FLT3* and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal hematopoietic cells but also leukemia cells (Ono, Nakajima et al. 2005; Zheng and

Small 2005; Levis 2011; Godfrey, Arora et al. 2012). The most common mutation of the *FLT3* gene is the *FLT3-ITD* mutation occurring in exons 14 and 15 and has been found in 15-35% of patients with AML and approximately 5% of patients with myelodisplasia (MDS). The presence of *FLT3*/ITD mutations is associated with decreased overall survival in both adult and pediatric AML patients (Rombouts, Blokland et al. 2000; Gilliland and Griffin 2002; Levis and Small 2003).

Mouse models have proven to be invaluable tools for understanding of human cancer. Nevertheless, significant differences between the genetic makeup of mice and humans make it difficult to directly extrapolate observations made in the former to a clinical disease in the latter. Unlike outbred human populations, mouse strains are genetically homogenous and homozygous across all most loci. The relative importance of a particular mutation or gene expression pattern to oncogenesis may be over- or underestimated in this context. Among others, for these reasons, normal primary human cells offer a potentially more relevant target for the study of oncogene function.

Human Embryonic Stem Cells (hESCs) are becoming a powerful tool for modeling human diseases in which pathogenesis and progression may not be fully recapitulated with the use of patient samples or mouse models. It is well known, that the process of leukemogenesis manifests as blockage of altered cell differentiation. Having this in mind, hematopoiesis-directed differentiation of hESCs could become a promising strategy to study the onset of hematopoiesis, especially the emergence of the earliest events leading to the specification of both normal and abnormal hematopoietic tissue (Lensch and Daley 2006). Previous studies made use of the BCR-ABL fusion gene to promote hematopoietic proliferation in mouse ESCs (Peters, Klucher et al. 2001; Bursen, Schwabe et al. 2010) and only a very recent study has explored the impact of HUP98-*HOX*A10 fusion oncogene on hESC-derived hematopoiesis (Ji, Risueno et al. 2011) . In 2012, Bueno et al. (Bueno, Montes et al. 2012) studied the effect of the expression of another *MLL* fusion gene (*MLL-AF4*) in human ESCs and its impact on the process of hematopoiesis-directed differentiation from hES cells. They showed that the expression of the *MLL-AF4* fusion gene in hESCs blocked the generation of $CD45^{+}$ cells and also strongly compromised the clonogenic potential of hematopoietic progenitors derived from hEB cultures.

Within this context, we were intrigued about the possible effects on the differentiation and proliferation processes of the *MLL-AF9*, the following most common fusion gene involving *MLL*. For addressing these questions, we aimed and we were successful at generating an immortalized hES cell line bearing the *MLL-AF9* fusion gene. This cellular reagent has never been produced in the scientific scenario. Therefore, this is the first study exploring the biological and developmental impact of

the *MLL-AF9* leukemic fusion gene on the onset of hematopoiesis from hES cells. In addition and more relevantly, we have also studied for the first time the effects of the co-expression of the *MLL-AF9* fusion oncogene and a known leukemic secondary hit *FLT3-ITD* on the process of hematopoiesis-directed differentiation of hES cells using different biological and functional assays.

5.2. THE BIOLOGICAL IMPACT OF THE *MLL-AF9* FUSION GENE AND THE MUTATED *FLT3-ITD* GENE

The main objective of our study was to create a human ESC model for the leukemic *MLL-AF9* fusion gene. We were successful to establish the first known immortal hESC line expressing this fusion oncogene. Moreover, we were capable to co-express in these cells a known leukemic secondary hit such as *FLT3-ITD*. These newly established hES cell lines served us for further biological and developmental studies such as hematopoietic differentiation, among others.

5.2.1. IMPACT ON THE HEMATOPOIESIS-DIRECTED DIFFERENTIATION OF hECSs

It is commonly known that the major consequence of the process of leukemogenesis is the blockage of hematopoietic differentiation. In our study we investigated the effects on the hESC hematopoietic induced differentiation that may be attributed to the expression of the *MLL-AF9* fusion gene. As we observed, the expression of the *MLL-AF9* fusion oncogene in hES cells showed only a slight effect on the early hemato-endothelial specification from hESCs. However, the *MLL-AF9* fusion gene induced late developmental defects in the hematopoietic lineage as shown by a highly reduced production of both $CD45^+$ and $CD45^+CD34^+$ hematopoietic cells (see Figures 4-17a-c). Similar data have been presented recently by Bueno et al. (Bueno, Montes et al. 2012), where in the case of the expression in hES cells of another leukemic fusion gene *MLL-AF4*, they also observed a significant blockage of hematopoietic differentiation of *MLL-AF4* hES cells.

Since the process of *in vitro* hematopoietic differentiation from hESCs closely mirrors early events in embryonic hematopoietic development, the results our study provide the first indication showing the capacity of the *MLL-AF9* leukemic fusion gene to impair embryonic blood formation when it is experimentally overexpressed in hESCs.

Our observations took a different perspective when we transformed the hESC model with a well-known oncogene. As can be seen in Figures 4-17e-f, when we introduced in the hESC model the ectopic expression of the *FLT3* genes (both the wild type (WT) and the ITD mutated copies), their expression apparently decreased the level of hemogenic precursors but, at the same time, increased the level of hematopoietic progenitors. As could be clearly observed upon the induction of the expression of *FLT3-WT*, this effect was probably due to an accelerated differentiation of hES cells into early committed primary hematopoietic cells rather than to an increased proliferation (confirmed by the cell cycle analysis). In the case of the *FLT3-ITD* expression, the levels of both hemogenic precursors and hematopoietic progenitors were lower, when compared with the hES cell line expressing the *FLT3-WT* form. This result is in line with previous reports, in which there has been shown that the overexpression of the *FLT3-ITD* mutant form can slightly block hematopoietic differentiation (Zheng, Friedman et al. 2004; Radomska, Basseres et al. 2006).

Accumulated data supports the notion that the leukemic cell population possesses a hierarchy of cells, with only a subset of them truly retaining the capability of self- renewal (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997). These leukemic stem cells (LSCs) are presumably necessary for the continued propagation of leukemia and therefore they represent the critical target cells for any successful therapy. Thus, genetic alterations that might affect LSC characteristics are of significant biological and clinical interest. Recent experiments have demonstrated that *MLL* fusion proteins including *MLL-AF9* are capable of imparting LSC-like properties on developing hematopoietic progenitors (Cozzio, Passegue et al. 2003; Krivtsov, Twomey et al. 2006; Somervaille and Cleary 2006). In 2008, we showed (Wei, Wunderlich et al. 2008) that the expression of the *MLL-AF9* fusion gene in human $CD34^+$ cells induced acute myeloid, lymphoid or mixed-lineage leukemia in immunodeficient mice with a media latency of 5-7 weeks. Our data suggested that some *MLL-AF9*-expresing LSCs were multipotent, while others were lineage restricted, demonstrating the heterogeneity of the LSCs in mixed leukemia. However, most recently, Bueno et al. (Bueno, Montes et al. 2012) has reported that the expression of the *MLL-AF4* fusion gene in hES cells was not sufficient to transform hESC-derived hematopoietic cells *in vitro* or *in vivo* suggesting the existence of a secondary leukemic hits, which are necessary and would lead to the development of leukemia. Similarly, in a recent report (Wang, Iwasaki et al. 2005), a conditional *MLL*-CBP knock-in mouse, that recapitulated the t-AML induced by the t(11;16) translocation, required chemical or irradiation-induced mutagenesis for the generation of a fatal myeloproliferative disease or leukemia. Furthermore, it become evident, even from the very early reports on the clonality, that bone marrow transplant

models progress to oligoclonal leukemias pointing to the need for cooperating mutations in *MLL*-induced leukemogenesis (Lavau, Szilvassy et al. 1997).

These results suggest that there is a need for cooperating secondary leukemic mutations to exist in order to develop acute myeloid leukemia. In such models of leukemogenesis, the initial genetic event often leads to the expression of chimeric fusion oncogenes encoded by recurrent chromosomal translocations, while subsequent mutations may activate specific signaling pathways (Dash and Gilliland 2001; Kelly and Gilliland 2002; Armstrong, Golub et al. 2003). Given that leukemias bearing translocations involving the *MLL* gene on chromosome 11q23 demonstrate high level expression and frequent mutation of the receptor tyrosine kinase FLT3, we hypothesized that the MLL-AF9 fusion protein and constitutively active FLT3 are cooperating genetic events in the multistep pathogenesis of *MLL-AF9* leukemia.

Recent hypotheses concerning the multistep nature of leukemogenesis suggest that the mutant signaling molecules are good candidates for the cooperation with DNA-binding fusion oncogenes during leukemogenesis (Gilliland and Griffin 2002). One mouse model indicates that this is indeed possible, as an activated receptor tyrosine kinase FLT3 induces an acute leukemia from an *MLL-SEPT6*-induced myeloproliferative disease (Ono, Nakajima et al. 2005). In another study, Stubbs et al. (Stubbs, Kim et al. 2008) showed in a mouse knock-in model that the simultaneous expression of the *NUP98-HOXD13* gene and the *FLT3-ITD* gene led to the development of acute leukemia with very short latency.

In 2005, Levis et al. (Levis, Murphy et al. 2005) showed that the *FLT3* mutations are present in human LSCs providing support for the importance of *FLT3* signaling in AML. Furthermore, it has been also demonstrated that high-level expression of *FLT3* is also associated with the presence of *MLL* translocations in both AML and ALL (Yeoh, Ross et al. 2002; Armstrong, Golub et al. 2003; Libura, Asnafi et al. 2003; Tsutsumi, Taketani et al. 2003). Moreover, Armstrong et al. (Armstrong, Kung et al. 2003) identified frequent mutation of *FLT3* in *MLL*-rearranged lymphoblastic leukemias, and demonstrated efficacy of a *FLT3* inhibitor against *MLL*-rearranged lymphoblastic leukemias in a xenograft model system. In 2008, Stubbs et al. (Stubbs, Kim et al. 2008) showed in a murine bone marrow model, that the *MLL-AF9* expression induced AML in approximately 70 days, whereas the combination of *MLL-AF9* and *FLT3-ITD* does so in less than 30 days. They showed also that the *MLL-AF9*/*FLT3-ITD*-induced leukemias were sensitive to *FLT3* inhibition in a 2-3 week *in vivo* assay confirming that the activated *FLT3* cooperated with *MLL-AF9* to accelerate the onset of AML from the whole bone marrow as well as a committed hematopoietic progenitors.

These mentioned mice and human data provided enough evidence about the

existence of the cooperation between the *MLL-AF9* and *FLT3* in the process of leukemogenesis.

Following this collaboration and trying to mimic what it has been shown in primary patients AML blasts, in our study, we have created for the first time a human ESC model expressing simultaneously the *MLL-AF9* and the *FLT3-WT*/*ITD* genes, which allowed us to investigate the effect of their co-expression on the onset of hematopoiesis from hES cells. As expected (see Fig 4-17h-i), the co-expression of the *MLL-AF9* and the *FLT3-ITD* genes in hES cells completely abrogated their hematopoietic potential. Similarly, the *MLL-AF9*/*FLT3-ITD* hESC-derived primary hematopoietic precursors showed significantly decreased clonogenic potential when compared to the control hES cell lines. Surprisingly, the co-expression of both oncogenes blocked also the early hemato-endothelial differentiation, as observed by very low levels of hemogenic precursors derived from hES cells. These results suggest that the co-expression of the *MLL-AF9* and the *FLT3-ITD* gene may exert a synergistic effect on the process of hematopoiesis-directed differentiation of hES cells and lead to the blockage at a very early level of hematopoietic differentiation.

Very surprising and unexpected were the results of the simultaneous co-expression of the *MLL-AF9* and the *FLT3-WT* gene in hES cells. The *MLL-AF9*/*FLT3-WT* hES cells, when subjected to hematopoiesis-directed differentiation, also showed a complete abrogation of their hematopoietic potential as observed by very low levels of hematopoietic progenitors and precursors. Based on the good onset of the hematopoietic differentiation observed on the *FLT3-WT*-expressing hES cell lines, we would expect that its co-expression with the *MLL-AF9* fusion gene in hES cells may at least partially recover their capacity to differentiate into hematopoietic cells and somehow overcome the blocking capacity of the *MLL-AF9* fusion gene. However this was not the case, the timecourse of the hematopoietic differentiation of *MLL-AF9*/*FLT3-WT* hES line was identical as for the *MLL-AF9*/*FLT3-ITD* hES cell line.

Despite being a leukemic oncogene, the *MLL-AF9* expression in hESCs did not seem to transform the cells *in vitro*. Unexpectedly, the *MLL-AF9* expressing hESCs did not display any proliferative survival as showed by the analysis of the cell cycle. These results are in line with the previous report by Bueno et al. (Bueno, Montes et al. 2012) where they reported that after the expression or another leukemic fusion gene *MLL-AF4* in hESCs, these cells did not acquire any survival or proliferation advantage.

Also an unexpected observation for us was the fact that, after the analysis of the cell cycle of *FLT3*-expressing hES cell lines, we did not observe any acquired proliferation advantage. It is commonly known that *FLT3* is normally expressed in hematopoietic stem/progenitor cells (HSPCs) and its expression is lost as

hematopoietic cells differentiate. It has been shown also that CD34 (a marker of primitive hematopoietic stem cells) is expressed prior to *FLT3* in hematopoietic progenitors (Adolfsson, Borge et al. 2001).

A large body of work has shown that *FLT3* plays significant roles in survival, proliferation and differentiation of hematopoietic cells (Lyman and Jacobsen 1998; Sakabe, Kimura et al. 1998; Gilliland and Griffin 2002; Levis and Small 2003). On the other hand, it has also been showed that *FLT3*-mediated responses are highly dependent on the cell type, and that other growth factors are acting on the cell in a well coordinated manner. Gabbianelli et al. (Gabbianelli, Pelosi et al. 1995) and others (Rusten, Lyman et al. 1996) have found that stimulation with *FLT3*L without the addition of other growth factors promoted the monocytic differentiation of early hematopoietic progenitor cells, without a marked proliferative response. However, FLT3L stimulation in combination with other growth factors such as interleukin-3 (IL-3), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), erythropoietin (EPO) and KIT ligand produced a more vigorous proliferative response (Shah, Smogorzewska et al. 1996) and greatly enhanced the development of granulocytic–monocytic colony-forming units (Gabbianelli, Pelosi et al. 1995; Rusten, Lyman et al. 1996). In our study, hESCs were transduced with lentiviral vectors only to over-express *FLT3-WT* and *FLT3-ITD* genes and then maintained in culture in an undifferentiated state without addition of any hematopoiesis-inducing cytokines. The combination of such cytokines (G-CSF, GM-CSF, BMP-4, EPO, TPO) was used only when the hES cells were subjected to hematopoietic differentiation using hEB protocol. This fact may be crucial in the understanding of the hematopoiesis-specified function of *FLT3* gene. In wild type undifferentiated hES cells, the level of *FLT3* expression is low and *FLT3* does not confer any proliferative advantage for these cells. In our *FLT3*-over-expressing hES cells, the level of endogenic *FLT3* was significantly higher (as expected) but did not play any important role in the proliferation of these cells. Nevertheless, the function of *FLT3* gene became of high importance when hES cells were subjected to hematopoiesis-directed differentiation. As we observed in the hematopoietic differentiation experiments, the cells expressing both the *FLT3-WT* and the *FLT3-ITD* forms, showed a higher levels of hematopoietic precursors and these primitive hematopoietic cells appeared earlier during hEB protocol when compared to the control cell lines. Based on published data and on our results, we could conclude that *FLT3* may act as an accelerator of hematopoietic differentiation of undifferentiated hES cells making them more susceptible for hematopoiesis-stimulating factors. Nevertheless, *FLT3* may also act as a stimulator of cell proliferation, but only in hematopoiesis-

committed cells. Further studies (such as analysis of the cell cycle and survival of hESC-derived hematopoietic progenitors) are required to understand completely the exact role of *FLT3* in hematopoiesis.

Concerning the chromosomal stability of newly established hES cell lines, it is worthy to highlight two major findings. By one hand, the mechanics of the *in vitro* culture introduced, at the very early passages, some aneuploidy (trisomies of chromosomes 12 and 20) (see Table 4-1) that was maintained as such along all passages and other genetic manipulations. On the other hand, when we introduced the lentiviral construct with both the WT and the ITD copies of *FLT3*, the karyotype analysis showed a structural rearrangement (a derivative of chromosome 10), which was also maintained as such along further manipulations. Since we observed the same aberration in two different experiments, we assumed that the chromosomal rearrangement was most likely the result of the transduction by itself rather than an effect due to the nature of the gene. Based on the results of the hematopoietic differentiation of hES cells expressing only *FLT3-WT* or *FLT3-ITD* gene we can conclude that these chromosomal changed did not negatively affected their hematopoietic potential. Whether these changes might contribute to the blockage of the hematopoietic differentiation in hES cells co-expressing the *MLL-AF9* and *FLT3-WT*/*FLT3-ITD* genes remains as an open question.

Summarizing, these data suggest that the hematopoiesis-accelerating function of *FLT3* gene may differ depending on the genetic context of the cell and may be altered (even annulled) in the presence of the *MLL-AF9* fusion gene. Undoubtly, further studies are required to better understand the mechanism of interaction between the *MLL-AF9* fusion gene and the *FLT3* gene and their cooperation in the development of acute myeloid leukemia. Once again, these results are in line with previous reports, in which it has been shown that the overexpression of the *FLT3-ITD* mutant form can slightly block hematopoietic differentiation (Zheng, Friedman et al. 2004; Radomska, Basseres et al. 2006).

5.2.2. IMPACT ON THE FUNCTIONALITY OF THE hESC-DERIVED HEMATOPOIETIC PRECURSOS

Hematopoiesis is the process in which stem cells divide and differentiate to produce the multiple types of mature cells found in blood. The process begins in early embryonic development and continues throughout adult life, primarily in the bone marrow. Various *in vivo* and *in vitro* assays have been developed to detect and assess the functionality of the stem cells and early multi-potential progenitors. Among them,

colony-forming cell (CFC) assays may be used to quantify lineage-restricted progenitors in a simple in vitro assay. When cultured in a semi-solid medium containing the appropriate cytokines, CFCs are able to divide and differentiate into a colony of more mature cells that can be detected by light microscopy. This allows for the quantification of erythroid, myeloid, lymphoid, megakaryocytic, and multi-potential cell lineages from various cell sources.

In 2010, Abdul-Nabi et al. (Abdul-Nabi, Yassin et al. 2010) showed in the CFC assay that the expression of the *MLL-AF9* fusion gene in primary human CD34+ cells caused erythroid hyperplasia and a clear block in the erythroid and myeloid maturation.

In our study, we decided to take advantage of the CFC assay and we characterized in such a way the biological properties of the hESC-derived hematopoietic precursors. We observed that the expression of the *MLL-AF9* fusion gene in hES cells strongly compromised the clonogenic potential of hematopoietic progenitors derived from day 15 of hEB culture (see Figure 4-18). These results are in line with recent study of Bueno et al. (Bueno, Montes et al. 2012), who also observed a strongly reduced clonogenic potential of hES cells expressing other leukemic fusion gene *MLL-AF4*.

In the case of hES cell lines expressing only the *FLT3-WT* we did not observe any reduction in the clonogenic potential of *FLT3-WT*-hESC-derived hematopoietic progenitors when compared with control hES cell lines as shown in the CFU assay. In the case of the FLT3-ITD hES cell line, we observed a slight decrease in the total number of colonies suggesting that the overexpression of the mutated form of the *FLT3* gene in hES cells might compromise the clonogenic potential of the *FLT3-ITD*-hESC-derived hematopoietic precursors. Our results are in line with previous report by Lee et al. (Lee, Tothova et al. 2007), who showed that the expression of the *FLT3-ITD* mutant gene in murine bone marrow caused a development of myeloproliferative disease with monocytic feature and which was the effect of disturbed clonogenic capacity of *FLT3-ITD*-positive hematopoietic precursors.

Summarizing, the expression of the *MLL-AF9* fusion gene (alone or together with *FLT3-WT*/*FLT3-ITD* genes) in hES cells compromised the clonogenic potential of hESC-derived hematopoietic precursors. The expression of the *FLT3-WT* gene or the *FLT3-ITD* gene alone might accelerate the process of hematopoietic differentiation of hES cells but does not improve the clonogenic capacity of the hES-derived hematopoietic precursors.

5.2.3. IMPACT ON THE EXPRESSION OF *MLL-AF9* TARGET GENES

MLL fusions are well-known positive regulators of homeobox gene expression (Bach, Buhl et al. 2010; Stam, Schneider et al. 2010). Aberrant expression of *HOX* genes definitively contributes to leukemogenesis (Buske and Humphries 2000). It has been shown that forced expression of *HOXA9* and *MEIS1* lead to the rapid development of myeloid leukemia in mice (Kroon, Krosl et al. 1998). It has been demonstrated that *HOXA9* and *MEIS1* suppress differentiation and maintain self-renewal of progenitor cells in response to growth factors (Calvo, Knoepfler et al. 2001; among others).

The patients with acute leukemias carrying *MLL* rearrangements show increased levels of expression of selected *HOX* genes (Imamura, Morimoto et al. 2002; Ferrando, Armstrong et al. 2003). It was also shown that the AML cells lines expressed the *HOX* genes and *MEIS1* gene significantly higher than the ALL cell lines (Quentmeier, Dirks et al. 2004). While in the case of the overexpression of the *FLT3-WT* and the *FLT3-ITD* genes, the quantitative analysis of the expression of selected *HOX* genes and *MEIS1* gene showed no change in their expression pattern when compared to the control hES cell lines; accordingly with these previous reports, in our study, we also observed significantly higher levels of selected *HOX* genes (*HOXA9*, *HOXB4*, *HOXB3*) and *MEIS1* gene in hES cells expressing the *MLL-AF9* fusion gene.

SUMMARY OF THE DISCUSSION

Our experimental approach has yielded a unique cellular model that provides a new system to study the cellular and molecular mechanisms underlying some of the *MLL-AF9*-mediated biological effects. Since the *MLL-AF9* expression does not alter the pluripotency, the unlimited replicative potential of hESCs enables the production of the *MLL-AF9*-expressing embryonic hematopoietic cells for studies that were previously unfeasible. Long-term scale, large-scale culture of *MLL-AF9* hESC-derived hematopoietic cells or hemogenic precursors provides an unprecedented system for drug screening and toxicity studies. It also offers a unique *in vitro* system to test the ability of potential cooperating oncogenic events or causal genotoxic compounds to induce leukemic transformation *in vitro.*

Taken together, our results provide the first indication showing how the leukemic fusion gene *MLL-AF9* when overexpressed in hESCs (separately or together with *FLT3* genes) impairs embryonic blood formation (Figure 5-1), establishing a potential novel experimental system to further study the developmental impact of the *MLL-AF9* fusion gene.

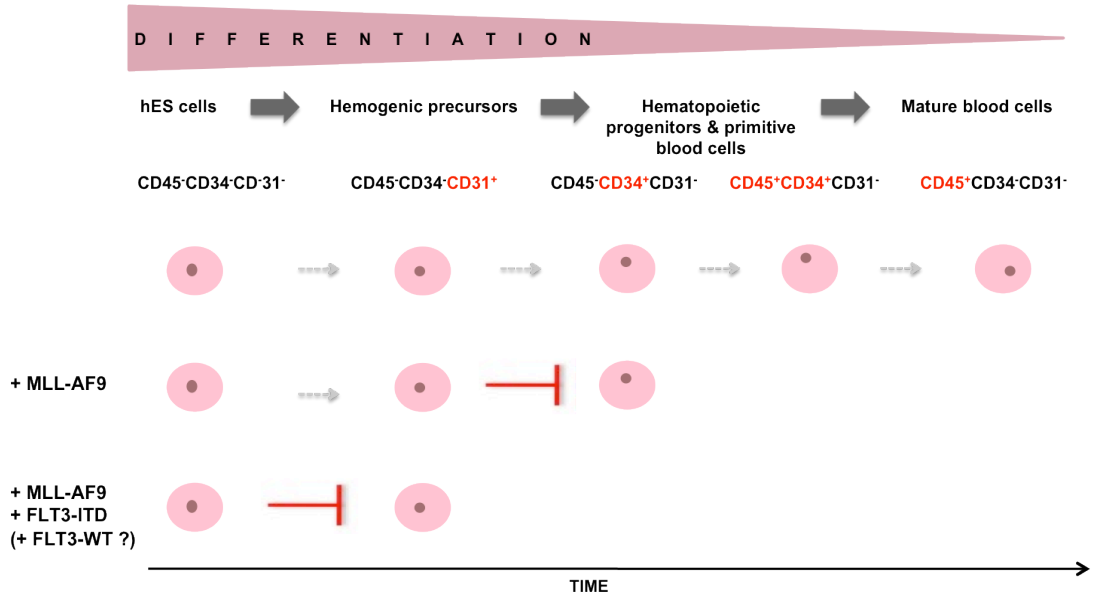

Figure 5-1 | Schematic representation of the proposed model of the leukemogenic impact of the *MLL-AF9* fusion gene and *FLT3* genes based on the results of hematopoietic differentiation from hES cells. The expression of the *MLL-AF9* oncogenic fusion gene slightly decrease the hemo-endothelial differentiation of hES cells but completely abrogates their further differentiation into hematopoietic precursors and mature blood cells. The simultaneous co-expression of the *MLL-AF9* fusion gene and the *FLT3* genes provokes a complete inhibition of the hematopoiesis-directed differentiation of hES cells already at its very early stage.

CONCLUSIONS

Conclusions to objective # 1:

- We have successfully established the first known human ESC model for the leukemic fusion gene *MLL-AF9*.
- The creation of the *MLL-AF9* hESC model allowed us to further expand its experimental potential by incorporating the co-expression of the secondary leukemogenic hit, the mutated *FLT3-ITD* gene.
- These newly established MLL-AF9-FLT3 hES cell lines became a unique cellular tool suitable for functional experiments, such as hematopoiesis-directed differentiation of hESC cells.

Conclusions to objective # 2:

- We have characterized some biological properties of the new transgenic hES cells. In detail:
- The newly established hESC lines expressing both the *MLL-AF9* fusion gene and the *FLT3* genes maintain their pluripotency state and the ability to differentiate into primitive hematopoietic cells, which enables the production of the *MLL-AF9-FLT3* hESC-derived hematopoietic cells for studies that were previously unfeasible.
- The expression of the *MLL-AF9* and *FLT3* oncogenes in human ES cells does not affect their proliferation capacity (does not confer any proliferation advantage), suggesting that these oncogenes are not sufficient to transform hES cells into leukemic stem cells *in vitro*.
- The expression of the *MLL-AF9* fusion gene in hES cells increases the expression levels of the potential *MLL-AF9* target *HOX* genes and the co-factor gene *MEIS1*, which fully reflects the situation observed in leukemic cells lines and in patient samples.
- The overexpression of the *FLT3-WT*/*FLT3-ITD* genes in hES cells may induce changes in their chromosomic stability, suggesting an additional function of *FLT3* gene in the process of leukemic transformation.

Conclusions for objective # 3:

- The expression of the *MLL-AF9* fusion gene in hES cells slightly decreases their hemo-endothelial differentiation capability and completely abrogates their potential to differentiate into hematopoietic cells. In addition, *MLL-AF9*-hESC-derived hematopoietic precursors posses a highly reduced clonogenic potential and ability to differentiate into mature blood cells. This recapitulates the situation observed in acute myeloid leukemia, where the process of hematopoiesis is disrupted by a blockage of hematopoietic differentiation.
- The overexpression of the *FLT3-WT/ITD* genes in hES cells may serve as an enhancer of hematopoietic differentiation. The absence of changes in the cell cycle of the *FLT3*-expresing hESC lines and, simultaneously, the increased number of hematopoietic precursors appearing earlier in the timecourse of the hematopoietic differentiation (when compared to the control hES cells lines) suggest that *FLT3* functions as an accelerator of the hematopoietic differentiation rather than an inductor of the proliferation of hES cells.
- The co-expression of an additional leukemogenic hit, such as *FLT3* Internal Tandem Duplication, together with the *MLL-AF9* fusion gene completely abrogates the hematopoietic differentiation potential of hES cells already at the very early stage of hemo-endothelial differentiation. Similarly to the *MLL-AF9* situation, *MLL-AF9-FLT3-ITD*-hESC-derived hematopoietic precursors manifest a highly reduced clonogenic potential and a complete abrogation of their ability to produce mature blood cells. This is also in line with the *MLL-AF9* AMLs samples, where the presence of the *FLT3-ITD* mutation is associated with shorter latency, more adverse effects and very poor prognosis of the patients.

CONCLUSIONES

Conclusiones del objetivo # 1:

- Hemos establecido con éxito el primer modelo humano conocido de células troncales humanas de naturaleza embrionaria con expresión del gen de fusión *MLL-AF9*.
- La creación del modelo del gen de fusión *MLL-AF9* nos permitió ampliar aún más su potencial experimental mediante la generación de un modelo celular derivado que incluye la co-expresión de otro evento genético leucemogénico, el gen mutado *FLT3-ITD*.
- Las líneas celulares establecidas MLL-AF9/FLT3 son en una herramienta única y útil para la realización de experimentos funcionales, por ejemplo, la diferenciación hematopoyética dirigida a partir de células troncales humanas de naturaleza embrionaria.

Conclusiones del objetivo # 2:

- Se han caracterizado algunas de las propiedades biológicas de las nuevas líneas celulares transgénicas de HES. En detalle:
- Las nuevas líneas celulares que expresan el gen de fusión *MLL-AF9* y el gen *FLT3* mantienen su estado de pluripotencia y la capacidad de diferenciarse en células primitivas hematopoyéticas. Esto permite la producción de las precursores tempranos hematopoyéticos que expresan la combinación oncogénica *MLL-AF9-FLT3* y su utilización estudios que anteriormente eran muy dificultosos de llevar a cabo.
- La expresión de los oncogenes *MLL-AF9* y *FLT3* en células troncales humanas de naturaleza embrionaria no afecta a no les confiere ventaja proliferativa, lo que sugiere que estos oncogenes no son suficientes para transformar dicho tipo celular embrionario en células leucémicas *in vitro*.
- La expresión del gen de fusión *MLL-AF9* en células troncales humanas de naturaleza embrionaria aumenta los niveles de expresión de algunos genes diana del gen de fusión *MLL-AF9*: genes del cluster *HOX* y el co-factor *MEIS1*, lo que refleja de parcialmente la situación que se observa en las líneas de células leucémicas y en muestras de pacientes.

Conclusiones para el objetivo # 3:

- La expresión del gen de fusión *MLL-AF9* en células troncales humanas de naturaleza embrionaria disminuye ligeramente su capacidad de diferenciación a precursores hemo-endoteliales y elimina completamente su potencial para diferenciarse en precursores hematopoyéticos. Además, estos precursores *MLL-AF9* han reducido su potencial clonogénico y su capacidad de diferenciarse en células sanguíneas maduras. Esto refleja la situación que se observa de forma general en los blastos de pacientes con leucemia mieloide aguda, donde el proceso de hematopoiesis está interrumpido por un bloqueo de la diferenciación hematopoyética.
- La sobreexpresión de los genes *FLT3-WT/FLT3-ITD* en las células troncales humanas de origen embrionario puede servir como un potenciador de la diferenciación hematopoyética. La ausencia de cambios en el ciclo celular de las líneas celulares FLT3 y simultáneamente, el mayor número de precursores hematopoyéticos que aparecen precozmente en el proceso de la diferenciación hematopoyética (en comparación con las células controles) sugieren que *FLT3* funciona más como un acelerador de la diferenciación hematopoyética que como un inductor de proliferación de células troncales humanas de naturaleza embrionaria.
- La co-expresión del encogen *FLT3-ITD* con el gen de fusión *MLL-AF9* elimina completamente el potencial de diferenciación hematopoyética de las células troncales humanas de origen embrionario, ya en una etapa muy temprana de la diferenciación de precursores hemo-endoteliales. De manera similar y más evidente a la situación que origina la expresión de *MLL-AF9*, los precursores hematopoyéticos derivados de las líneas MLL-AF9-FLT3-ITD manifiestan de forma muy reducida su potencial clonogénico y una eliminación completa de su capacidad de producir células sanguíneas maduras. Esto también está en consonancia con las muestras de *MLL-AF9* AML, en las que la presencia de la mutación *FLT3-ITD* se asocia con menor latencia, efectos más adversos y muy mal pronóstico de los pacientes.

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ANNEX

MEDIAN
CD45-CD34+CD31+

	day 3	day 7	day 10	day 15
AND2	0,4	0,84	8,67	9,57
AND2-NEO	0,62	0,58	7,3	8,44
AND2-MLL-AF9	0,43	0,49	4,34	5,2

	day 3	day 7	day 10	day 15
AND2-NEO-FLT3-WT	0,14	1,103	6,57	6,56
AND2-NEO-FLT3-ITD	0,06	2,2	3,93	2,53

	day 3	day 7	day 10	day 15
AND2-MLL-AF9-FLT3-WT	0,13	1,24	1,07	1,47
AND2-MLL-AF9-FLT3-ITD	0,03	1,11	0,81	0,64

SD
CD45-CD34+CD31+

	day 3	day 7	day 10	day 15
AND2-WT	0,035118846	0,746970196	0,8326664	0,280685929
AND2-NEO	0,260256284	0,149331845	1,086640837	1,02600284
Neo+WT	0,229227689	0,361985267	0,404145188	1,179898987
Neo+ITD	0,04	1,131320792	1,289702808	1,144812547
AND2-MAF9	0,752484773	0,21007935	1,166756187	1,000555504
MAF9+WT	0,147422296	1,127001019	0,967281414	1,102589931
MAF9+ITD	0,040066611	1,004058431	1,123046452	1,089190678

MEDIAN
CD45+CD34+CD31-

	day 3	day 7	day 10	day 15
AND2	0,54	0,64	1,9	2,41
AND2-NEO	0,67	0,65	1,63	1,75
AND2-MLL-AF9	0,22	0,48	0,45	0,57

	day 3	day 7	day 10	day 15
AND2-NEO-FLT3-WT	0,03	3,13	5,73	5,7
AND2-NEO-FLT3-ITD	0,09	1,78	3,96	3,26

	day 3	day 7	day 10	day 15
AND2-MLL-AF9-FLT3-WT	0,17	0,94	1,1	1,14
AND2-MLL-AF9-FLT3-ITD	0,05	0,71	0,82	0,37

SD
CD45+CD34+CD31-

	day 3	day 7	day 10	day 15
AND2-WT	0,0521664	0,550124376	0,152752523	0,384602006
AND2-NEO	0,120968315	0,470567034	0,37320075	0,720489648
Neo+WT	0,035851546	0,450924975	1,253490693	1,106179035
Neo+ITD	0,100166528	1,10138843	1,150308607	1,155233939
AND2-MAF9	0,174355958	0,352325607	0,479404581	0,147676448
MAF9+WT	0,147309199	1,048568606	1	0,665832812
MAF9+ITD	0,031505438	0,80158177	1,1075348	0,345976878

MEDIAN
CD45+CD34-CD31-

	day 3	day 7	day 10	day 15
AND2	0,4	0,36	1,14	3,52
AND2-NEO	0,44	0,2	0,83	2,78
AND2-MLL-AF9	0,34	0,12	0,24	0,22

	day 3	day 7	day 10	day 15
AND2-NEO-FLT3-WT	1,1	1,14	1,23	1,96
AND2-NEO-FLT3-ITD	0,63	0,4	0,46	1,15

	day 3	day 7	day 10	day 15
AND2-MLL-AF9-FLT3-WT	0,31	0,41	0,37	0,48
AND2-MLL-AF9-FLT3-ITD	0,27	0,179	0,11	0,22

SD
CD45+CD34-CD31-

	day 3	day 7	day 10	day 15
AND2-WT	0,641565328	0,447695116	0,098149546	0,504706375
AND2-NEO	0,467653718	0,233880311	0,62171805	0,711776503
Neo+WT	0,414889157	0,2136196	0,460827625	0,493288286
Neo+ITD	0,785820101	0,51507281	0,73050211	0,215715862
AND2-MAF9	0,415812458	0,200748599	0,337194306	0,274500152
MAF9+WT	0,612673853	0,421465697	0,057735027	0,2
MAF9+ITD	0,446790033	0,502008964	0,101488916	0,141421356

Figure S-1 | Detailed data from the hematopoietic differentiaion analysis. Presented data are median form 3 different experiments ± SEM.

ALL CELL LINES	MEDIAN	SD
AND2	10,4	1,290994449
AND2-NEO	9,3	0,577350269
AND2-NEO-FLT3-WT	11,25	0,957427108
AND2-NEO-FLT3-ITD	7,25	2,081665999
AND2-MLL-AF9	3	0,577350269
AND2-MLL-AF9-FLT3-WT	1,5	0,577350269
AND2-MLL-AF9-FLT3-ITD	1,25	0,957427108

Figure S-2 | Deatiled counts from the CFU assay. Presented data are median from 3 different experiments ± SEM.

Cloning of Human Hematopoietic Stem Cell specific gene promoters

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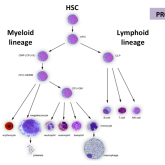
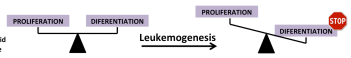
INTRODUCTION

HEMATOPOIESIS AND ACUTE MYELOID LEUKEMIA

Hematopoiesis is a highly regulated process where decisions have to be made on whether the Hematopoietic Stem Cells (HSC) should self-renew, (proliferate) or differentiate into the different blood cells.



Schema of the hematopoietic differentiation

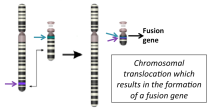


In **Acute Myeloid Leukemia**, the balance among proliferation and differentiation is disrupted, leading to bone marrow failure. Recurrent **chromosomal rearrangements** that result in the production of fusion proteins are common initiation events in this hematological malignancy.

OBJETIVE

To find some gene promoters which will be active only in human hematopoietic stem cells (hHSC, CD34 (+) cells), in order to achieve the conditional expression of different fusion genes with known leukemic potential, during hematopoietic process.

The involvement of the **fusion genes** in the **hematopoietic differentiation blockage** has been partially studied but when and how does it occurs needs to be further explored.



Chromosomal translocation which results in the formation of a fusion gene

SELECTECTION OF hHSC SPECIFIC GENES

LMO2	It is required for yolk sac erythropoiesis and has a central and crucial role in hematopoietic development.
GATA 2	It has an essential role in regulating transcription of genes involved in the development and proliferation of hematopoietic and endocrine cell lineages.
TAL1	It may play an important role in hematopoiesis process, specially as a positive regulator of erythroid differentiation. It is implicated in the genesis of hemopoietic malignancies.
HOXB4	It is is a member of the Antp homeobox family. Intracellular or ectopic expression of this protein expands hematopoietic stem and progenitor cells in vivo and in vitro.

MATERIAL & METHODS

VECTORS

1.Lentiviral vector: pLVX-AcGFP-N1-PGK-Puro

2. Non viral vector: pEGFP- N1

QUANTITATIVE PCR

The literature attributes the selected promoters a high hematopoietic specificity, but we must demonstrate this **expression profile** in our cellular models, by qPCR .

CELLULAR MODELS

CD34(+) CELLS	CD34(-) CELLS
CD34(+) fraction from Cord Blood	CD34(-) fraction from Cord Blood
KASUMI 1 cell line	NAMALWA1 cell line
K562 cell line	293FT cell line

ELECTROPORATION AND LENTIVIRAL TRANDUCCTION

ELECTROPORATION

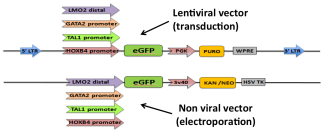
To perform a transient "in vitro" validation of the promoter activity in the selected cellular models.

LENTIVIRAL TRANSDUCTION

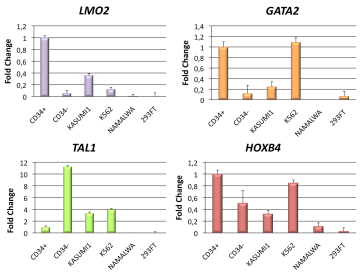
To establish long-term cultures of CD34+ cells which will allow us to evaluate the expression pattern of the promoters along the hematopoietic differentiation process.

RESULTS

1. Cloning of hHSC specific promoters…

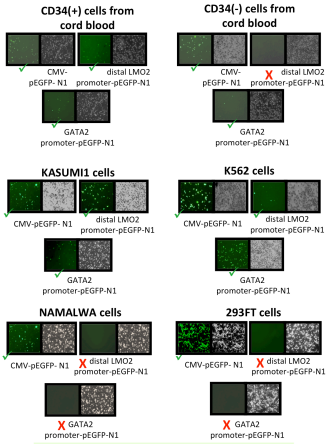


2. qPCR validation: selected genes expression level



According to these results, we decided to continue the study only with *LMO2* and *GATA 2* promoters.

3. Electroporation results in the cellular models



We validated the qPCR results by electroporating the different cellular models. Again, the specificity of both promoters seems quite obvious.

CONCLUSIONS

1. qPCR results gave us quite satisfactory with ***LMO2*** and ***GATA2*** genes. However, *TAL1* and *HOXB4* do not seem to be so specific to HSC.
2. GFP expression under the control of *LMO2* distal promoter and *GATA2* was observed in K562, Kasumi1 and CD34(+) cells, but not in CD34(-) cells. Those findings confirm the **specificity** of the two promoters to **hematopoietic stem cells**. (Long-term assay with lentiviral transductions is still ongoing)
3. We are providing a **system for the functional analysis of human CD34+HSC**, which will give us the opportunity to compare the effect of the constitutive expression and conditional expression of different leukemic chimeric protein.

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Figure S-3 | Poster presented at the Meeting of the International Society of Cellular Oncology (ISCO), March 4-8, 2012, Mallorca, Spain.

Modelling leukemic fusion gene MLL-AF9 in human Embryonic Stem Cells

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ABSTRACT

Leukemias harboring MLL-AF9 fusion protein are associated with a dismal prognosis and their clinical consequences are well characterized.
Concerning the biological processes affected by these fusion gene a little is known and our understanding of its transformation capacities is limited. It is not known at which stage of hematopoietic differentiation this translocations can occur and which is the critical one in the process of leukemiogenesis.
Human Embryonic Stem Cells (hESCs) are becoming a powerful tool for modelling human diseases in which pathogenesis and progression may not be fully recapitulated with the use of patient samples or mouse models.
Here we explored *in vitro* the impact of the enforced expression of MLL-AF9 and FLT3 genes on hematopoietic differentiation from human Embryonic Stem Cells.
Preliminary results of hematopoietic-directed differentiation of hESCs indicate that the expression of MLL-AF9 decreases the onset of CD34+ HSCs by blocking hematopoiesis at very early stage. Moreover, the clonogenic potential of these hematopoietic precursors is also reduced as shown by CFU assay. Nevertheless, further studies are still carried out to better characterize the leukemogenic potential of this fusion gene.

OBJECTIVES

In our study we decided to develop a model of leukemic fusion gene MLL-AF9 in hESCs. These model will provide unique tool to investigate its effects on the process of hematopoietic differentiation and to characterize the genomics and epigenomics associated with this particular oncogenic pathway.

INTRODUCTION & WORKING HYPOTHESIS

MLL gene (Fig.1a) is a histone methyltransferase acting as a positive regulator of gene transcription and is expressed in hematopoietic cells (stem and progenitor cells).

Fig.1a

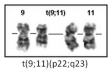

Fig.1b

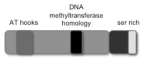

Fig.1c

MLL rearrangements are found in >70% of infant leukemias. MLL translocations are also found in approximately 10% of AML in adults and in therapy-related leukemias. One of the most frequent translocation found in leukemias is t(9;11)(p22;q23) (Fig.1b) which leads to MLL-AF9 fusion gene (Fig.1c). [1,3]

FLT3, a member of the receptor tyrosine kinase (RTK) class III, is preferentially expressed on the surface of a high proportion of acute myeloid leukemia (AML) and B-lineage acute lymphocytic leukemia (ALL). A mutated form of FLT3 gene, FLT3-ITD has been found in approximately 30% of adult AML patients and in approximately 10% of childhood AML patients. An interaction of FLT3 and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal hematopoietic cells but also leukemia cells. [2]

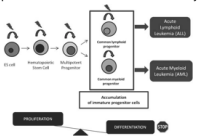

Fig.2a

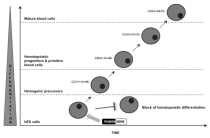

Fig.2b

The process affected by leukemic translocations is hematopoiesis. In case of normal hematopoiesis, hematopoietic stem cells (HSCs) originated from embryonic stem cells (ESCs), posses the ability of self-reneval and proliferation and can further differentiate into lymphoid and myeloid progenitors , giving rise to mature blood cells. There is a balance between proliferation and differentiation.
When the hematopoiesis gets disrupted by oncogenic events, there is an accumulation of immature/malignant progenitor cells, increased proliferation and block of differentiation that may leads to the development of leukemia (Fig.2a,b).
In our study we hypothesize that the expression of MLL-AF9 fusion gene in hESCs inhibits their hematopoietic-directed differentiation, which in the presence of known secondary hits (e.g. FLT3 mutation) may lead to leukemogenic transformation of hematopoietic stem cells.

RESULTS

Establishment of transgenic hES cell line

Human ES cell line AND2 (Fig.4) was transduced with lentiviral vector (LV) bearing MLL-AF9 fusion gene (Fig.3).

Fig.3

48h after LV transduction a selection with G418 was performed (during 4 weeks) and the integration and expression of MLL-AF9 fusion has been confirmed by qRT-PCR (Fig.4).

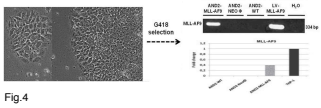

Fig.4

Newly established cell lines was subsequently transduced with LV bearing WT and mutated form of FLT3 gene (Fig.5).

Fig.5

The integration of FLT3 gene has been confirmed by RT-PCR (Fig.6).

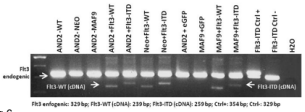

Fig.6

The pluripotency state of newly generated cells lines has been confirmed by analysis of expression of pluripotency markers OCT4, TRA-1-81 (Fig.7).

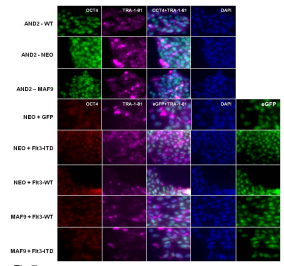

Fig.7

Hematopoietic differentiation from hES cells

Hematopoietic-directed differentiation was performed with wild type and transgene AND2 cell lines using a standard protocol based on Embryonic Body formation and 7- day culture with special cytokine cocktail (Fig.8). [4]

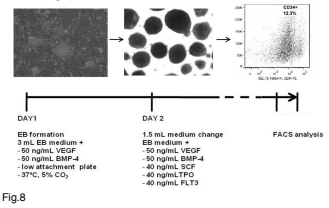

Fig.8

The efficiency of hematopoietic differentiation was compared in wild type cells, cells bearing empty LV vector and cells expressing MLL-AF9 fusion and both WT and mutated FLT3 gene. FACS analysis was performed at four different timepoints . Data are presented as a mean ± SEM from three independent experiments (Fig.9).

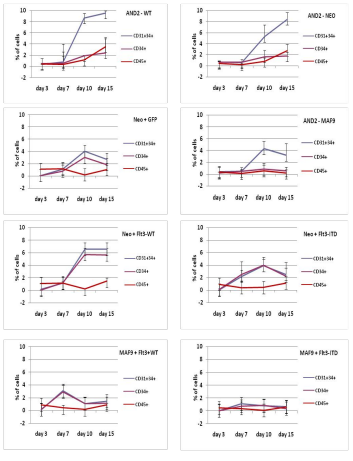

Fig.9

The clonogenic potential of hESC-derived CD34+ hematopoietic progenitors was measured by the ability to form colony-forming units (CFUs) in semisolid culture. Data are presented as a mean ± SEM from four independent experiments (Fig.10).

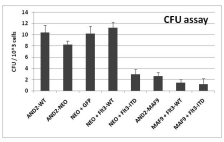

Fig.10

The morphology of observed colonies was typical for myeloid lineages (macrophage and granulocyte-macrophage) (Fig.11).

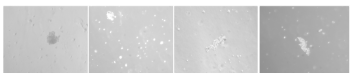

Fig.11

CONCLUSIONS

1. New transgenic hES cell lines expressing MLL-AF9 fusion gene and FLT3 genes have been established and their pluripotency state has been confirmed by expression of pluripotency genes (RT-PCR; IF).
2. It has been demonstrated that the expression of both oncogenes (MLL-AF9 and FLT3-ITD) affects the onset of hESC-derived hematopoietic progenitors indicating the blockage of hematopoietic-directed differentiation.
3. A significant difference in the clonogenic potential was observed between hematopoietic progenitors derived from WT and transgenic hESCs .
4. Further studies are still carrying out to better characterize the leukemogenic potential of MLL-AF9 fusion gene.

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Figure S-4 | Poster presented at the 7th CBCTTC Brazilian Congress on Stem Cells and Cell Therapy, October 3-6, 2012, Sao Paulo, Brazil.

Fundación Centro Nacional de Investigaciones Oncológicas Carlos III

LA FUNDACIÓN JESÚS SERRA PRESENTA EN EL CNIO SU PROYECTO DE AYUDA A LA INVESTIGACIÓN ONCOLÓGICA

Madrid, 2 de noviembre, 2012. La Fundación Jesús Serra, perteneciente al Grupo Catalana Occidente, ha entregado esta semana al Centro Nacional de Investigaciones Oncológicas (CNIO) sus ayudas de investigación, dirigidas a impulsar proyectos científicos innovadores y de alto impacto, durante un acto que se celebró en el Auditorio del CNIO.

El Programa de Investigadores visitantes Fundación Jesús Serra busca la colaboración de eminentes especialistas internacionales con el equipo de investigación del CNIO durante unos meses de estancia para profundizar y aprovechar sinergias.

El tercer beneficiario de las ayudas es Jarowslaw Sochacki, que se encuentra completando su doctorado en el Grupo de Citogenética Molecular, dirigido por Juan Cruz Cigudosa.

El acto contó con la presencia de Federico Halpern, presidente de la Fundación Jesús Serra, Erwin Wagner, además de los tres investigadores beneficiarios de las ayudas, los respectivos jefes de grupo y Marisol Quintero, directora de Innovación del CNIO.

Los investigadores beneficiarios de las ayudas junto a los jefes de grupo del CNIO y los representantes de la Fundación Jesús Serra asistentes al acto. **CNIO**

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